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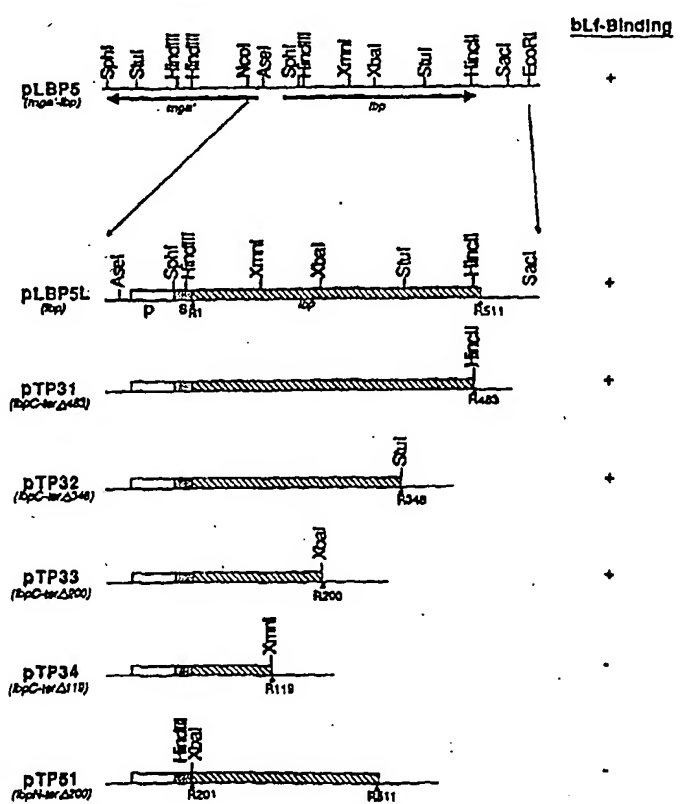
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(54) Title: *STREPTOCOCCUS UBERIS* LACTOFERRIN–BINDING PROTEIN

(57) Abstract

The bovine lactoferrin (bLF) binding protein of *Streptococcus uberis (S. uberis)* is described, as well as the gene encoding the bLF (*lbp*). LF–binding proteins can be used in vaccine compositions for the prevention and treatment of *S. uberis* infections, particularly mastitis, as well as in diagnostic methods for determining the presence of *S. uberis* infections. Also disclosed is a regulatory region adjacent to *lbp*, termed *mga*.

5   *STREPTOCOCCUS UBERIS* LACTOFERRIN-BINDING PROTEIN

Technical Field
        The present invention relates generally to
10   bacterial antigens. More particularly, the present
     invention pertains to the characterization and
     recombinant production of a bovine lactoferrin-binding
     protein from *Streptococcus uberis* (*S. uberis*) and the
     use of the same. The invention also relates to the
15   characterization of a regulatory region, *mga*, located
     upstream of the lactoferrin-binding protein gene.

Background
        Mastitis, an infection of the mammary gland,
20   causes major economic losses to the dairy industry
     yearly. *Streptococcus uberis* (*S. uberis*) is an
     environmental pathogen responsible for a high
     proportion of cases of mastitis in lactating cows and
     is the predominant organism isolated from mammary
25   glands during the nonlactating period (Bramley, A.J.
     (1984) *Br. Vet. J.* 140:328-335; Bramley and Dodd
     (1984) *J. Dairy Res.* 51:481-512; Oliver, S.P. (1988)
     Am. J. Vet. Res. 49:1789-1793). Mastitis resulting
     from infection with *S. uberis* is commonly subclinical,
30   characterized by apparently normal milk with an
     increase in somatic cell counts due to the influx of
     leukocytes. The chemical composition of milk is
     changed due to suppression of secretion with the
     transfer of sodium chloride and bicarbonate from blood
35   to milk, causing a shift of pH to a more alkaline
     level. *S. uberis* mastitis may also take the form of

an acute clinical condition, with obvious signs of
disease such as clots or discoloration of the milk and
swelling or hardness of the mammary gland. Some cases
of the clinical disease can be severe and pyrexia may
5   be present. For a review of the clinical
manifestations of *S. uberis* mastitis, see, Bramley
(1991) Mastitis: physiology or pathology. p. 3-9. In
C. Burvenich, G. Vandeputte-van Messom, and A. W. Hill
(ed.), *New insights into the pathogenesis of mastitis.*
10   Rijksuniversiteit Gent, Belgium; and Schalm et al.
(1971) The mastitis complex-A brief summary. p. 1-3.
In *Bovine Mastitis.* Lea & Febiger, Philadelphia.

The pathogenesis of *S. uberis* infection is
poorly understood. Furthermore, the influence of *S.*
15   *uberis* virulence factors on host defense mechanisms
and mammary gland physiology is not well defined.
Known virulence factors associated with *S. uberis*
include a hyaluronic acid capsule, hyaluronidase, R-
like protein, plasminogen activator and CAMP factor.
20   However, very little is known of their roles in
pathogenicity.

Lactoferrin (Lf) is a mammalian iron-binding
glycoprotein secreted by polymorphonuclear leukocytes
(PMNs) and various exocrine glands (Baggiolini et al.
25   (1970) *J. Exp. Med.* 131:559-570; Masson et al. (1966)
Clin. Chim. Acta 14:735-739). This protein is found
at high concentrations in milk and at mucosal surfaces
(Masson et al., *supra;* Reiter and Oram (1967) *Nature*
216:328-330). For example, bovine lactoferrin (bLf)
30   concentrations in lacteal secretions can increase up
to 30-fold during acute bovine mastitis, depending on
the severity of infection (Harmon et al. (1976)
Infect. Immun. 13:533-542).

A regulatory function for Lf in various
35   physiological pathways, including the adhesion of PMNs
to the endothelial surface, feedback inhibition of the

granulocyte-monocyte colony-stimulating factor, and the regulation of antibody production, has been suggested. Specific interaction of Lf with certain mammalian cells seems to be involved in the above pathways and specific receptors for Lf have been identified on macrophages, monocytes, B lymphocytes, PMNs, activated T lymphocytes, and hepatocytes (Bennet and Davis (1981) *J. Immunol.* 127:1211-1216; Dehanne et al. (1985) *Am. J. Physiol.* 248:463-469; Maneva et al. (1983) *Int. J. Biochem.* 15:981-984; Rochard et al. (1989) *FEBS Lett.* 255:201-204; and van Snick and Masson (1976) *J. Exp. Med.* 144:1568-1580).

Lf inhibits the growth of *E. coli* and certain other microorganisms *in vitro* (Bullen et al. (1972) *Br. Med. J.* 1:69-75). This Lf-mediated antimicrobial action has mainly been attributed to its iron deprivation capacity with bacteria (Arnold et al. (1977) *Science* 197:263-265; Law and Reiter (1977) *J. Dairy Res.* 44:595-599; Oram and Reiter (1968) *Biochim. Biophys. Acta* 170:351-365). In this regard, it is well known that with few exceptions, iron is essential for microbial growth (Weinberg, E.D. (1978) *Microbiol. Rev.* 42:45-66. Even though iron is abundant within mammalian tissues, virtually all iron within the mammalian body is held intracellularly as ferritin or as heme compounds, pools which are generally inaccessible to invading microorganisms. Additionally, the small amount of iron present in extracellular spaces is effectively chelated by high-affinity iron-binding host glycoproteins such as transferrin (Tf), present in serum and lymph, and Lf, present in secretory fluids and milk (Otto et al. (1992) *Crit. Rev. Microbiol.* 18:217-233).

To overcome this deficiency, bacterial pathogens have developed specific iron uptake mechanisms. In many bacterial species, these

mechanisms involve the synthesis and secretion of
small compounds called siderophores which display high
affinity for ferric iron (FeIII). Siderophores are
capable of removing TF- or Lf-bound iron to form
5   ferrisiderophore complexes which in turn are
recognized by specific iron-repressible membrane
receptors and internalized into the bacterium where
the iron is released (Crosa, J.H. (1989) *Microbiol.
Rev.* 53:517-530). This iron uptake mechanism has been
10   described for many gram-negative bacterial species.
Some gram-negative bacteria do not secrete detectable
siderophores when grown in an iron-deficient
environment but produce outer membrane proteins that
bind directly and specifically to Tf or Lf, thereby
15   allowing iron transport into the bacterial cell.

Tf binding appears to be mediated by the
activity of two proteins present in bacterial outer
membranes, transferrin-binding protein 1 and 2 (Tbp1
and Tbp2) (Gonzalez et al. (1990) *Mol. Microbiol.*
20   4:1173-1179; Ogunnariwo and Schryvers (1990) *Infect.
Immun.* 58:2091-2097; Schryvers, A.B. (1989) *J. Med.
Microbiol.* 29:121-130); Schryvers and Lee (1989) *Can.
J. Microbiol.* 35:409-415; Schryvers and Morris (1988)
Mol. Microbiol. 2:467-472). Transferrin binding
25   proteins tend to be highly specific for the
transferrin of their natural host.

However, the mechanism of iron uptake from
Lf has not been well characterized. A putative 105
kDa receptor for Lf utilization, Lbp1, has been
30   identified in gonococcus by affinity isolation
(Schryvers and Lee, *supra*; Cornelissen et al. (1992)
J. Bacteriol. 174:5788-5797; Lee and Bryan (1989) *J.
Med. Microbiol.* 28:199-204). The structural gene for
Lbp1, termed *lbpA*, has been isolated (Biswas and
35   Sparling (1995) *Infect. Immun.* 63:2958-2967). The
genes for meningococcal lactoferrin receptors have

also been characterized (Petterson et al. (1993)
Infect. Immun. 61:4724-4733; Petterson et al. (1994)
J. Bacteriol. 176:1764-1766; Petterson et al. (1994)
Microb. Pathog. 17:395-408). The DNA sequence of *lbpA*

5   and the predicted amino acid sequence of Lbp1 in
gonococcus and the meningococcus are highly conserved
(94% identity). Lbp1 has been shown to be 46%
identical to Tbp1 (Cornelissen et al. (1992) *J.
Bacteriol.* 174:5788-5797) of the same gonococcal

10   strain but only 18% identical to Tbp2 (Anderson et al.
(1994) *J. Bacteriol.* 176:3162-3170). Both gonococcal
and meningococcal genes contain relatively well-
conserved Fur boxes and the proteins are homologous to
the TonB-dependent family of receptors, as is true for

15   Tbp1 (Cornelissen et al., *supra*) but not for Tbp2
(Anderson et al., *supra*). The strong similarity
between the Lf receptor protein, Lbp1, and the Tf
receptor protein, Tbp1, suggests that binding of Lf to
bacterial cells might be similar to Tf binding.

20   Consistent with this hypothesis is the fact that the
putative protein encoded by *lbpB*, the open reading
frame upstream of *lbpA*, shows extensive homology to
Tbp2 (Pettersson et al., (1994) *Microb. Pathog.*
17:395-408), suggesting that iron-acquisition from Lf,

25   as from Tf, requires two specific proteins in the
outer membrane.

     In contrast to the knowledge of the iron
uptake systems of Gram-negative bacteria, there is
comparatively little information concerning the

30   mechanisms by which Gram-positive pathogens acquire
iron during growth in extracellular body fluids. Both
S. aureus and the coagulase-negative staphylococci
have been reported to produce siderophores
(Konetschny-Rapp et al. (1991) *Eur. J. Biochem.*

35   191:65-74; Meiwes et al. (1990) *FEMS Microbiol. Lett.*
67:201-206). *S. aureus* appears capable of binding

both human and bovine Lfs and human Tf (Naidu et al.
(1991) *J. Med. Micrbiol.* 34:323-328; Naidu et al.
(1991) *J. Dairy Sci.* 74:1218-1226; Naidu et al. (1992)
J. Med. Microbiol. 36:177-183; Modun et al. (1994)

5   *Infect. Immun.* 62:3850-3858). The interaction of Lf
with a bovine *S. agalactiae* strain has also been
reported (Rainard, P. (1992) *FEMS Microbiol. Lett.*
98:235-240). However, the iron acquisition functions
of these Tf- or Lf-binding proteins have not been

10 studied.

The group A streptococcal M protein is
considered to be one of the major virulence factors of
this organism by virtue of its ability to impede
attack by human phagocytes (Lancefield, R.C. (1962) *J.*

15 *Immunol.* 89:307-313). The bacteria persist in the
infected tissue until antibodies are produced against
the M molecule. Type-specific antibodies to the M
protein are able to reverse the antiphagocytic effect
of the molecule and allow efficient clearance of the

20 invading organism. For example, M proteins are one of
the key virulence factors of *S. pyogenes*, due to their
involvement in mediating resistance to phagocytosis
(Kehoe, M.A. (1991) *Vaccine* 9:797-806) and their
ability to induce potentially harmful host immune

25 responses via their superantigenicity and their
capacity to induce host-cross-reactive antibody
responses (Bisno, A.L. (1991) *New Engl. J. Med.*
325:783-793; Froude et al. (1989) *Curr. Top.*
Microbiol. Immunol. 145:5-26; Stollerman, G.H. (1991)

30 *Clin. Immunol. Immunopathol.* 61:131-142).

In group A streptococci (GAS), the genes for
M protein (*emm*) as well as a peptidase (*scpA*) and, if
present, genes encoding M protein-related IgG- and
IgA-binding proteins (*fcrA* and *enn*, respectively) are

35 clustered on the chromosome (Haanes et al. (1992) *J.*
Bacteriol. 174:4967-4976; Hollingshead et al. (1993)

Mol. Microbiol. 8:707-717; Podbielski, A. (1993) *Mol. Gen. Genet.* 237:287-300). Expression of these virulence-associated surface proteins is co-regulated at the level of transcription by the protein Mga (which stands for multigene regulator of group A Streptococcus), formerly called Mry or VirR (Caparon and Scott (1987) *Proc. Natl. Acad. Sci. USA* 84:8677-8681; Chen et al. (1993) *Mol. Gen. Genet.* 241:685-693; Haanes and Cleary (1989) *J. Bacteriol.* 171:6397-6408; McIver et al. (1995) *J. Bacteriol.* 177:6619-6624; Perez-Casal et al. (1991) *J. Bacteriol.* 173:2617-2624; Podbielski et al. (1995) *Infect. Immun.* 63:9-20; Podbielski, A. (1992) *Med. Microbiol. Immunol.* 181:227-240; Robbins et al. (1987) *J. Bacteriol.* 169:5633-5640). It is thought that Mga is a part of a crucial regulatory system in GAS, possibly functioning as a second component in a two-component regulatory system.

Vaccination is one approach to enhance resistance of the mammary gland to new infection and reduce clinical severity of the disease. Previous studies have shown that primary infection with *S. uberis* can considerably reduce the rate of infection following a second challenge with the same strain (Hill, A.W. (1988) *Res.Vet. Sci.* 44:386-387). Local vaccination with killed *S. uberis* protects the bovine mammary gland against intramammary challenge with the homologous strain (Finch et al. (1994) *Infect. Immun.* 62:3599-3603). Similarly, subcutaneous vaccination with live *S. uberis* has been shown to cause a dramatic modification of the pathogenesis of mastitis with the same strain (Hill et al. (1994) *FEMS Immunol. Med. Microbiol.* 8:109-118). Animals vaccinated in this way shed fewer bacteria in their milk and many quarters remain free of infection.

However, vaccination with live or attenuated bacteria can pose risks to the recipient. It would therefore be desirable to provide a subunit vaccine composition for use against *S. uberis*. Until now, the

5   *S. uberis* lactoferrin-binding protein has not been characterized and it use in vaccine compositions has not been described.

Disclosure of the Invention

10   The present invention is based on the discovery of a bovine lactoferrin (bLF) binding protein (bLbp) from *S. uberis*, and the characterization thereof. The gene coding for bLF-binding protein, *lbp*, as well as an upstream regulator

15   of the gene, *mga*, have been cloned. bLF-binding protein, immunogenic fragments and analogs thereof, and/or chimeric proteins including the same, can be used, either alone or in combination with other antigens, in novel subunit vaccines to provide

20   protection from bacterial infection in mammalian subjects.

Accordingly, in one embodiment, the subject invention is directed to an isolated, immunogenic *S. uberis* bLF-binding protein, as well as a nucleic acid

25   molecule comprising a coding sequence for an immunogenic *S. uberis* bLF-binding protein. In additional embodiments, the invention is directed to recombinant vectors including the same, host cells transformed with these vectors and methods of

30   recombinantly producing *S. uberis* bLF-binding proteins.

In still further embodiments, the subject invention is directed to vaccine compositions comprising a pharmaceutically acceptable vehicle and

35   an immunogenic *S. uberis* bLF-binding protein.

In yet other embodiments, the present invention is directed to methods of treating or preventing *S. uberis* infections, such as mastitis, in a mammalian subject. The method comprises

5   administering to the subject a therapeutically effective amount of the above vaccine compositions.

In additional embodiments, the invention pertains to methods of producing vaccine compositions comprising (a) providing an immunogenic *S. uberis* bLF-

10   binding protein; and (b) combining the protein with a pharmaceutically acceptable vehicle.

In further embodiments, the invention is directed to antibodies against the S. uberis bLF-binding proteins.

15   In additional embodiments, the invention is directed to methods of detecting *S. uberis* antibodies in a biological sample comprising:

(a) providing a biological sample;

(b) reacting the biological sample with a *S.*

20   *uberis* bLF-binding protein under conditions which allow *S. uberis* antibodies, when present in the biological sample, to bind to the *S. uberis* bLF-binding protein to form an antibody/antigen complex; and

25   (c) detecting the presence or absence of the complex,

thereby detecting the presence or absence of *S. uberis* antibodies in the sample.

In yet further embodiments, the invention is

30   directed to an immunodiagnostic test kit for detecting *S. uberis* infection. The test kit comprises a *S. uberis* bLF-binding protein and instructions for conducting the immunodiagnostic test.

In further embodiments, the invention is

35   directed to an isolated *S. uberis* Mga protein, as well

as a nucleic acid molecule comprising a coding
sequence for the same.

These and other embodiments of the present
invention will readily occur to those of ordinary

5   skill in the art in view of the disclosure herein.

Brief Description of the Figures

Figure 1 is a restriction enzyme map of *lbp*
and shows progressive deletions and a summary of bLf

10   binding data. Open boxes (p region) represent 5'-
sequences containing promoter and ribosome binding
sites, shaded boxes (s region) represent lbp sequences
encoding the signal peptides, and hatched boxes
represent the *lbp* sequences coding for the mature or

15   truncated proteins. R1 and R201 (R represents
residue) indicate the first codon of the mature
protein in pLBP5 and the truncated protein in pTP51,
respectively. Other numbers indicate the last codon
of each protein. Restriction sites are present on the

20   top of the *lbp*. The bLf binding ability of each clone
is shown on the right.

Figures 2A-2C (SEQ ID NOS:1-2) depict the
nucleotide sequence and deduced amino acid sequence of
S. uberis bovine Lbp. Nucleotides and amino acids are

25   numbered on the right of the sequences. The deduced
amino acid sequence is shown in the single-letter code
below the nucleotide sequence. Two possible ATG start
codons at positions 232 and 262, and the TAA stop
codon at 1915, are shown in bold. Two putative -35

30   and -10 promoter sequences and Shine-Dalgarno
sequences (SD) are indicated. A putative rho-
independent transcription terminator (T) is
underlined. The double underline shows the presence
of a putative signal peptide at the N-terminus of the

35   ORF. The C-terminal hydrophobic trans-membrane domain
is indicated by italics and the nearby surface anchor

-10-

motif is shown by italics and double underline. The
central repeated amino acid sequences are indicated by
the letters A, B, and C.

5   Figure 3 shows profiles of the secondary
structures, charged residues and hydrophobicity of
Lbp. The deduced amino acid sequence of Lbp was
analyzed with the Novotny-Auffray algorithm. Plots
marked Turn, Beta, and Alpha indicate the potential
for beta turn-random coil, beta sheet, and alpha helix
10   formation, respectively. The +/- plot shows regions
of the molecule with net positive (upper) and negative
(lower) charges. The hydrophobicity (Hydro) plot
shows the hydrophobic regions of the protein. The
positions of the amino acids are shown on the
15   horizontal axis.

Figure 4 depicts the construction of pMGA14F
from pLBP5i and pMGA14. Plasmid pMGA14F was generated
by inserting the 1.5 kb *SphI-NheI* fragment of pLBP5i
into the *SphI* and *NheI* sites of pMGA14. Lines
20   indicate *S. uberis* DNA, while the box represents the
multiple cloning sites of vector pTZ18R. The probe
fragments used for Southern or Northern blot
experiments are indicated by the hatched bars. The
arrows indicate the locations of the open reading
25   frames of *lbp*, *mga'* and *mga*.

Figures 5A-5D (SEQ ID NOS:3-12) show the
nucleotide sequence of *mga* and deduced amino acid
sequence of Mga, as well as the ORFs downstream of
mga. Nucleotides and amino acids are numbered on the
30   right of the sequences. The deduced amino acid
sequence is shown in the single-letter code below the
nucleotide sequence. Possible ATG start codons are
shown in bold and the stop codons are indicated by
"*". A putative -35 and -10 promoter sequence and
35   Shine-Dalgarno sequence (SD) are indicated.

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Figure 6 shows the probes used in
hybridization analysis, as well as the structure of
Lbp (on the top), constructed from the DNA sequence
analysis. The signal peptide, proline rich region and

5   transmembrane domain are indicated by S, Pro and TM
respectively. The A, B and C repeat regions are also
shown. Probes used in hybridization are indicated by
the hatched bars below the map.

Figure 7 shows the time course of ^{125}I-bLf

10   binding to *S. uberis*. 10^9 bacteria were incubated with
6.9 nm ^{125}I-bLf in 0.2 ml of PBS-1% BSA. At time
intervals indicated, bacteria were pelleted and the
amount of cell associated ^{125}I-bLf was determined.

Figure 8 depicts the results of a

15   competition binding assay using bLf (33% iron-
saturated) as radiolabelled ligand and competitor.
Percentage binding values were calculated as
percentage of ^{125}I-bLf binding in the presence of
increasing amounts of unlabelled bLf to bacteria

20   suspended in PBS-1% BSA in the absence of unlabelled
bLf. Inset: Scatchard plot and affinity (Kd) of the
binding of ^{125}I-bLf to *S. uberis*. The line represents
the best fit as determined by a linear regression
analysis. A concentration of 270 nM of unlabelled bLf

25   caused 50% displacement of ^{125}I-bLf binding (indicated
by dotted lines).

Figure 9 shows the influence of iron
chelators on the expression of lactoferrin-binding by
S. uberis. Cells grown in THB-YE with or without

30   EDDA, dipyridyl or desferrioxamine mesylate were
incubated with 6.9 nM ^{125}I-bLf in 0.2 ml of PBS-1% BSA
at room temperature for 2 h. After three washes,
cell-bound radioactivity was determined.

Figure 10 shows the physical map of the

35   recombinant plasmid used to express the Lbp in Example
3. The plasmid pLBP5 contains 3.7 kb of *S. uberis-*

derived kDNA, in the vector pTZ18R. The plasmid pGH-
LBP was constructed by subcloning an *SphI-RsaI*
fragment from pLBP5 into vector pGH433. P$_{tac}$ indicates
the location of the *tac* promoter. The Lbp gene is
5   shown by the arrows labelled as *lbp*.

Figure 11 shows inhibition of recombinant
bLf-binding protein on ^{125}I-labelled bLf binding to *S.
uberis*. Increasing amounts of a mixture of supernatant
(sup.) and whole cell lysate (with equal volume) of *E.
10   coli* pLBP5 (●) or *E. coli* pTZ18R (O) were mixed with
10^9 cells and incubated with 0.69 nM ^{125}I-bLf in 0.2 ml
volumes. Inhibition values were calculated as relative
percentage of bLf binding to bacteria suspended in
PBS-1% BSA in the absence of any *E. coli* samples.
15

Detailed Description

The practice of the present invention will
employ, unless otherwise indicated, conventional
techniques of molecular biology, microbiology,
20   recombinant DNA technology, and immunology, which are
within the skill of the art. Such techniques are
explained fully in the literature. See, e.g.,
Sambrook, Fritsch & Maniatis, *Molecular Cloning: A
Laboratory Manual*, Vols. I, II and III, Second Edition
25   (1989); *DNA Cloning*, Vols. I and II (D.N. Glover ed.
1985); *Oligonucleotide Synthesis* (M.J. Gait ed.
1984); *Nucleic Acid Hybridization* (B.D. Hames & S.J.
Higgins eds. 1984); *Animal Cell Culture* (R.K.
Freshney ed. 1986); *Immobilized Cells and Enzymes*
30   (IRL press, 1986); Perbal, B., *A Practical Guide to
Molecular Cloning* (1984); the series, *Methods In
Enzymology* (S. Colowick and N. Kaplan eds., Academic
Press, Inc.); and *Handbook of Experimental Immunology*,
Vols. I-IV (D.M. Weir and C.C. Blackwell eds.,
35   1986, Blackwell Scientific Publications).

A. Definitions

        In describing the present invention, the
following terms will be employed, and are intended to
be defined as indicated below.

5   It must be noted that, as used in this
specification and the appended claims, the singular
forms "a", "an" and "the" include plural referents
unless the content clearly dictates otherwise. Thus,
for example, reference to "an Lbp" includes a mixture
10 of two or more Lbps, and the like.
        The terms "lactoferrin-binding protein",
"LF-binding protein" and "Lbp" (used interchangeably
herein) or a nucleotide sequence encoding the same,
intends a protein or a nucleotide sequence,
15 respectively, which is derived from an *S. uberis lbp*
gene. The nucleotide sequence of a representative *S.
uberis lbp* gene, and the corresponding amino acid
sequence of an LF-binding protein encoded by this
gene, are depicted in Figures 2A-2C (SEQ ID NOS:1-2).
20 However, an LF-binding protein as defined herein is
not limited to the depicted sequence as several
subtypes of *S. uberis* are known and variations in LF-
binding proteins will occur between strains of *S.
uberis*.
25   Furthermore, the derived protein or
nucleotide sequences need not be physically derived
from the gene described above, but may be generated in
any manner, including for example, chemical synthesis,
isolation (e.g., from *S. uberis*) or by recombinant
30 production, based on the information provided herein.
Additionally, the term intends proteins having amino
acid sequences substantially homologous (as defined
below) to contiguous amino acid sequences encoded by
the genes, which display immunological and/or
35 lactoferrin-binding activity.

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Thus, the terms intend full-length, as well
as immunogenic, truncated and partial sequences, and
active analogs and precursor forms of the proteins.
Also included in the term are nucleotide fragments of
5   the gene that include at least about 8 contiguous base
pairs, more preferably at least about 10-20 contiguous
base pairs, and most preferably at least about 25 to
50 or more contiguous base pairs of the gene. Such
fragments are useful as probes and in diagnostic
10   methods, discussed more fully below.
The terms also include those forms
possessing, as well as lacking, the signal sequence,
as well as the nucleic acid sequences coding therefor.
Additionally, the term intends forms of LF-binding
15   protein which lack the membrane anchor region, and
nucleic acid sequences encoding such deletions. Such
deletions may be desirable in systems that do not
provide for secretion of the protein. Furthermore, an
LF-binding domain, found within about the N-terminal
20   200 codons, may or may not be present. Thus, for
example, if the Lf binding protein will be used to
purify LF, the LF-binding domain will generally be
retained. If the protein is to be used in vaccine
compositions, immunogenic epitopes which may or may
25   not include the LF-binding domain, will be present.
The terms also include proteins in neutral
form or in the form of basic or acid addition salts
depending on the mode of preparation. Such acid
addition salts may involve free amino groups and basic
30   salts may be formed with free carboxyls.
Pharmaceutically acceptable basic and acid addition
salts are discussed further below. In addition, the
proteins may be modified by combination with other
biological materials such as lipids (both those
35   occurring naturally with the molecule or other lipids
that do not destroy immunological activity) and

saccharides, or by side chain modification, such as acetylation of amino groups, phosphorylation of hydroxyl side chains, oxidation of sulfhydryl groups, glycosylation of amino acid residues, as well as other
5   modifications of the encoded primary sequence.

The term therefore intends deletions, additions and substitutions to the sequence, so long as the polypeptide functions to produce an immunological response as defined herein. In this
10   regard, particularly preferred substitutions will generally be conservative in nature, i.e., those substitutions that take place within a family of amino acids. For example, amino acids are generally divided into four families: (1) acidic -- aspartate and
15   glutamate; (2) basic -- lysine, arginine, histidine; (3) non-polar -- alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar -- glycine, asparagine, glutamine, cystine, serine threonine, tyrosine. Phenylalanine,
20   tryptophan, and tyrosine are sometimes classified as aromatic amino acids. For example, it is reasonably predictable that an isolated replacement of leucine with isoleucine or valine, or vice versa; an aspartate with a glutamate or vice versa; a threonine with a
25   serine or vice versa; or a similar conservative replacement of an amino acid with a structurally related amino acid, will not have a major effect on the biological activity. Proteins having substantially the same amino acid sequence as the
30   reference molecule, but possessing minor amino acid substitutions that do not substantially affect the immunogenicity of the protein, are therefore within the definition of the reference polypeptide.

By "mastitis" is meant an inflammation of
35   the mammary gland in mammals, including in cows, ewes, goats, sows, mares, and the like, caused by the

presence of *S. uberis*. The infection manifests itself
by the infiltration of phagocytic cells in the gland.
Generally, 4 clinical types of mastitis are
recognized:.(1) peracute, associated with swelling,

5   heat, pain, and abnormal secretion in the gland and
accompanied by fever and other signs of systemic
disturbance, such as marked depression, rapid weak
pulse, sunken eyes, weakness and complete anorexia;
(2) acute, with changes in the gland similar-to those

10   above but where fever, anorexia and depression are
slight to moderate; (3) subacute, where no systemic
changes are displayed and the changes in the gland and
its secretion are less marked: and (4) subclinical,
where the inflammatory reaction is detectable only by

15   standard tests for mastitis.

          Standard tests for the detection of mastitis
include but are not limited to, the California
Mastitis Test, the Wisconsin Mastitis Test, the Nagase
test, the electronic cell count and.somatic cell

20   counts used to detect a persistently high white blood
cell content in milk. In general, a somatic cell
count of about 300,000 to about 500,000 cells per ml
or higher, in milk will indicate the presence of
infection. Thus, a vaccine is considered effective in

25   the treatment and/or prevention of mastitis when, for
example, the somatic cell count in milk is retained
below about 500,000 cells per ml. For a discussion of
mastitis and the diagnosis thereof, see, e.g., *The
Merck Veterinary Manual. A Handbook of Diagnosis,*

30   *Therapy, and Disease Prevention and Control for the
Veterinarian,* Merck and Co., Rahway, New Jersey, 1991.

          An "isolated" nucleic acid molecule is a
nucleic acid molecule separate and discrete from the
whole organism with which the molecule is found in

35   nature; or a nucleic acid molecule devoid, in whole or
part, of sequences normally associated with it in

nature; or a sequence, as it exists in nature, but having heterologous sequences (as defined below) in association therewith.

By "subunit vaccine composition" is meant a
5   composition containing at least one immunogenic polypeptide, but not all antigens, derived from or homologous to an antigen from a pathogen of interest. Such a composition is substantially free of intact pathogen cells or particles, or the lysate of such
10   cells or particles. Thus, a "subunit antigen composition" is prepared from at least partially purified (preferably substantially purified) immunogenic polypeptides from the pathogen, or recombinant analogs thereof. A subunit antigen
15   composition can comprise the subunit antigen or antigens of interest substantially free of other antigens or polypeptides from the pathogen.

The term "epitope" refers to the site on an -antigen or hapten to which specific B cells and/or T
20   cells respond. The term is also used interchangeably with "antigenic determinant" or "antigenic determinant site." Antibodies that recognize the same epitope can be identified in a simple immunoassay showing the ability of one antibody to block the binding of
25   another antibody to a target antigen.

An "immunological response" to a composition or vaccine is the development in the host of a cellular and/ or antibody-mediated immune response to the composition or vaccine of interest. Usually, an
30   "immunological response" includes but is not limited to one or more of the following effects: the production of antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells and/or $\gamma\delta$ T cells, directed specifically to an antigen or
35   antigens included in the composition or vaccine of interest. Preferably, the host will display either a

therapeutic or protective immunological response such
that resistance of the mammary gland to new infection
will be enhanced and/or the clinical severity of the
disease reduced. Such protection will be demonstrated

5   by either a reduction or lack of symptoms normally
displayed by an infected host, a quicker recovery time
and/or a lowered somatic cell count in milk from the
infected quarter.

        The terms "immunogenic" protein or

10   polypeptide refer to an amino acid sequence which
elicits an immunological response as described above.
An "immunogenic" protein or polypeptide, as used
herein, includes the full-length sequence of the LF-
binding protein, with or without the signal sequence,

15   membrane anchor domain and/or LF-binding domain,
analogs thereof, or immunogenic fragments thereof. By
"immunogenic fragment" is meant a fragment of an LF-
binding protein which includes one or more epitopes
and thus elicits the immunological response described

20   above. Such fragments can be identified using any
number of epitope mapping techniques, well known in
the art. See, e.g., *Epitope Mapping Protocols* in
Methods in Molecular Biology, Vol. 66 (Glenn E.
Morris, Ed., 1996) Humana Press, Totowa, New Jersey.

25   For example, linear epitopes may be determined by
e.g., concurrently synthesizing large numbers of
peptides on solid supports, the peptides corresponding
to portions of the protein molecule, and reacting the
peptides with antibodies while the peptides are still

30   attached to the supports. Such techniques are known
in the art and described in, e.g., U.S. Patent No.
4,708,871; Geysen et al. (1984) *Proc. Natl. Acad.*
Sci. USA 81:3998-4002; Geysen et al. (1986) *Molec.*
Immunol. 23:709-715. Similarly, conformational

35   epitopes are readily identified by determining spatial
conformation of amino acids such as by, e.g., x-ray

crystallography and 2-dimensional nuclear magnetic
resonance.   See, e.g., *Epitope Mapping Protocols*,
supra.

5   Immunogenic fragments, for purposes of the
present invention, will usually include at least about
3 amino acids, preferably at least about 5 amino
acids, more preferably at least about 10-15 amino
acids, and most preferably 25 or more amino acids, of
the Lbp molecule.   There is no critical upper limit to
10   the length of the fragment, which could comprise
nearly the full-length of the protein sequence, or
even a fusion protein comprising two or more epitopes
of Lbp.

          "Native" proteins or polypeptides refer to
15   proteins or polypeptides isolated from the source in
which the proteins naturally occur.   "Recombinant"
polypeptides refer to polypeptides produced by
recombinant DNA techniques; i.e., produced from cells
transformed by an exogenous DNA construct encoding the
20   desired polypeptide.   "Synthetic" polypeptides are
those prepared by chemical synthesis.

          A "vector" is a replicon, such as a plasmid,
phage, or cosmid, to which another DNA segment may be
attached so as to bring about the replication of the
25   attached segment.

          A DNA "coding sequence" or a "nucleotide
sequence encoding" a particular protein, is a DNA
sequence which is transcribed and translated into a
polypeptide *in vitro* or *in vivo* when placed under the
30   control of appropriate regulatory elements.   The
boundaries of the coding sequence are determined by a
start codon at the 5' (amino) terminus and a
translation stop codon at the 3' (carboxy) terminus.
A coding sequence can include, but is not limited to,
35   procaryotic sequences, cDNA from eucaryotic mRNA,
genomic DNA sequences from eucaryotic (e.g.,

mammalian) DNA, and even synthetic DNA sequences. A
transcription termination sequence will usually be
located 3' to the coding sequence.

5   DNA "control elements" refers collectively
to promoters, ribosome binding sites, polyadenylation
signals, transcription termination sequences, upstream
regulatory domains, enhancers, and the like, which
collectively provide for the transcription and
translation of a coding sequence in a host cell. Not
10   all of these control sequences need always be present
in a recombinant vector so long as the desired gene is
capable of being transcribed and translated.

         "Operably linked" refers to an arrangement
of elements wherein the components so described are
15   configured so as to perform their usual function.
Thus, control elements operably linked to a coding
sequence are capable of effecting the expression of
the coding sequence. The control elements need not be
contiguous with the coding sequence, so long as they
20   function to direct the expression thereof. Thus, for
example, intervening untranslated yet transcribed
sequences can be present between a promoter and the
coding sequence and the promoter can still be
considered "operably linked" to the coding sequence.

25   A control element, such as a promoter,
"directs the transcription" of a coding sequence in a
cell when RNA polymerase will bind the promoter and
transcribe the coding sequence into mRNA, which is
then translated into the polypeptide encoded by the
30   coding sequence.

         A "host cell" is a cell which has been
transformed, or is capable of transformation, by an
exogenous nucleic acid molecule.

         A cell has been "transformed" by exogenous
35   DNA when such exogenous DNA has been introduced inside
the cell membrane. Exogenous DNA may or may not be

integrated (covalently linked) into chromosomal DNA
making up the genome of the cell. In procaryotes and
yeasts, for example, the exogenous DNA may be
maintained on an episomal element, such as a plasmid.

5   With respect to eucaryotic cells, a stably transformed
cell is one in which the exogenous DNA has become
integrated into the chromosome so that it is inherited
by daughter cells through chromosome replication.
This stability is demonstrated by the ability of the

10   eucaryotic cell to establish cell lines or clones
comprised of a population of daughter cells containing
the exogenous DNA.

"Homology" refers to the percent identity
between two polynucleotide or two polypeptide

15   moieties. The correspondence between the sequence
from one moiety to another can be determined by
techniques known in the art. For example, homology
can be determined by a direct comparison of the
sequence information between two polypeptide molecules

20   by aligning the sequence information and using readily
available computer programs such as ALIGN, Dayhoff,
M.O. (1978) in *Atlas of Protein Sequence and
Structure* 5:Suppl. 3, National biomedical Research
Foundation, Washington, DC.

25   Alternatively, homology can be determined by
hybridization of polynucleotides under conditions
which form stable duplexes between homologous regions,
followed by digestion with single-stranded-specific
nuclease(s), and size determination of the digested

30   fragments. Two DNA, or two polypeptide sequences are
"substantially homologous" to each other when the
sequences exhibit at least about 80%-85%, preferably
at least about 90%, and most preferably at least about
95%-98% sequence identity over a defined length of the

35   molecules, as determined using the methods above. As
used herein, substantially homologous also refers to

sequences showing complete identity to the specified
DNA or polypeptide sequence. DNA sequences that are
substantially homologous can be identified in a
Southern hybridization experiment under, for example,
5   stringent conditions, as defined for that particular
system. Defining appropriate hybridization conditions
is within the skill of the art. See, e.g., Sambrook
et al., *supra*; *DNA Cloning*, *supra*; *Nucleic Acid
Hybridization*, *supra*.

10   The term "functionally equivalent" intends
that the amino acid sequence of an LF-binding protein
is one that will elicit a substantially equivalent or
enhanced immunological response, as defined above, as
compared to the response elicited by an LF-binding
15   protein having identity with the reference LF-binding
protein, or an immunogenic portion thereof.

A "heterologous" region of a DNA construct
is an identifiable segment of DNA within or attached
to another DNA molecule that is not found in
20   association with the other molecule in nature. Thus,
when the heterologous region encodes a bacterial gene,
the gene will usually be flanked by DNA that does not
flank the bacterial gene in the genome of the source
bacteria. Another example of the heterologous coding
25   sequence is a construct where the coding sequence
itself is not found in nature (e.g., synthetic
sequences having codons different from the native
gene). Allelic variation or naturally occurring
mutational events do not give rise to a heterologous
30   region of DNA, as used herein.

The term "treatment" as used herein refers
to either (i) the prevention of infection or
reinfection (prophylaxis), or (ii) the reduction or
elimination of symptoms of the disease of interest
35   (therapy).

As used herein, a "biological sample" refers
to a sample of tissue or fluid isolated from a
subject, including but not limited to, for example,
blood, plasma, serum, fecal matter, urine, bone

5 marrow, bile, spinal fluid, lymph fluid, samples of
the skin, external secretions of the skin,
respiratory, intestinal, and genitourinary tracts,
tears, saliva, milk, blood cells, organs, biopsies and
also samples of *in vitro* cell culture constituents

10 including but not limited to conditioned media
resulting from the growth of cells and tissues in
culture medium, e.g., recombinant cells, and cell
components.

As used herein, the terms "label" and

15 "detectable label" refer to a molecule capable of
detection, including, but not limited to, radioactive
isotopes, fluorescers, chemiluminescers, enzymes,
enzyme substrates, enzyme cofactors, enzyme
inhibitors, chromophores, dyes, metal ions, metal

20 sols, ligands (e.g., biotin or haptens) and the like.
The term "fluorescer" refers to a substance or a
portion thereof which is capable of exhibiting
fluorescence in the detectable range. Particular
examples of labels which may be used under the

25 invention include fluorescein, rhodamine, dansyl,
umbelliferone, Texas red, luminol, NADPH and α-β-
galactosidase.

B.   General Methods

30   Central to the present invention is the
discovery of a bovine LF-binding protein in *S. uberis*.
The gene for the *S. uberis* bLF-binding protein ("*lbp*")
has been isolated and characterized, and the protein
encoded thereby sequenced. The complete DNA and amino

35 acid sequences of *S. uberis* bLF-binding protein are
shown in Figures 2A-2C (SEQ ID NOS:1-2). In

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particular, as described in the examples, a single ORF
of 1683 bp, depicted as residues 232-1914, inclusive
of Figures 2A-2C (SEQ ID NOS:1-2), encoding 561 amino
acid residues, gives rise to two protein species able
5   to bind bovine lactoferrin, having molecular weights
of 76 kDa and 165 kDa, respectively. The 165 kDa
protein is likely a dimer of the 76 kDa protein since
urea treatment results in a single band and Northern
blot analysis shows only one major transcript in *S.*
10   *uberis*, as well as in recombinant *E. coli* transformed
with a construct encoding the LF-binding protein.

S. *uberis* bovine LF-binding protein includes
a putative N-terminal signal peptide of about 50 amino
acids (if translation starts at the first ATG codon
15   shown in Figure 2A). Thus, the full-length bovine LF-
binding protein depicted, including the signal
sequence, is found at amino acid positions 1-561,
inclusive, (encoded by nucleotide positions 232-1914,
inclusive) of Figures 2A-2C (SEQ ID NOS:1-2). The
20   mature protein, lacking the signal peptide, is found
at amino acid positions 52-561, inclusive, (nucleotide
positions 445-1914, inclusive) of Figures 2A-2C. A
membrane anchor motif at the C-terminus is also
present, as indicated in Figures 2A-2C (SEQ ID NOS:1-
25   2). A bovine Lf binding domain is present in a 200
codon N-terminal region of the molecule. The protein
appears to lack disulfide bridges.

As shown in the examples, the binding of
^{125}I-bLf to S. *uberis* was time-dependent and
30   displaceable by unlabelled bLf. Apo-bLf inhibits ^{125}I-
bLf binding as effectively as iron-saturated bLf.
Bovine transferrin, human lactoferrin and human
transferrin do not interfere with bLf binding. The
Scatchard plot is linear and approximately 7800
35   binding sites are expressed by each bacterial cell,
with an affinity of 1.0 x 10^{-7} M. Reduced iron

availability does not significantly modify the
saturation of *S. uberis* by bLf. The bLf binding
protein described herein is lactoferrin species-
specific, in that human Lf does not appear to
5   effectively block the binding of bovine Lf.

        The Lf binding protein of *S. uberis* differs
from the transferrin receptors of *Haemophilus* and
Neisseria spp., which consist of two distinct
transferrin-binding proteins, termed Tbp1 and Tbp2,
10   which range in molecular weight from 68 to 105 kDa
depending on the strain. Similarly, the bovine Lf
receptor of *S. aureus* consists of two distinct bLf
binding proteins with estimated molecular weights of
92 and 67 kDa (Naidu et al. (1991) *J. Dairy Sci.*
15   <u>74</u>:1218-1226) and therefore appears to be different
from the receptor described herein. Also, the
streptococcal LF-binding protein described herein
appears to be different from the *S. aureus* human Lf
binding protein, an approximately 450 kDa protein
20   which, under reducing SDS-PAGE gel conditions,
resolves into two components of 67 and 62 kDa.

        Analysis of the primary and secondary
structure of the *S. uberis* bLf binding protein
suggests that it is an M-like protein. In particular,
25   a gene homologous to the group A streptococcal *mga*, a
positive regulator of M and M-like proteins, has been
found in the upstream adjacent region of *lbp*.
Southern blot analysis reveals that *mga* is present in
all *S. uberis* strains tested that contained the *lbp*.

30   The sequence of *S. uberis mga* and the
protein product therefrom is presented in Figures 5A-
5D (SEQ ID NOS:3-12). Starting at the ATG initiation
codon at nucleotides 361-363 and terminating at a TAA
codon at nucleotides 1858-1860, the deduced gene
35   product, Mga, is comprised of 499 amino acid residues
with a calculated molecular weight of 58,454 Da. The

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N-terminus of Mga lacks the features of a signal
peptide, suggesting that it is a cytoplasmic protein.
Preceding the start codon of *mga* is a putative
ribosome binding site AGGAGA. Sequences resembling
5   the -35 and -10 promoter motifs have also been
identified, as shown in Figures 5A-5D (SEQ ID
NOS:3-12).

S. uberis LF-binding protein, immunogenic
fragments thereof or chimeric proteins including the
10   same, can be provided in subunit vaccine compositions
to treat or prevent bacterial infections caused by *S.
uberis*, including mastitis in mammals, such as in
bovine, equine, ovine and goat species. In addition
to use in vaccine compositions, the proteins and
15   fragments thereof, antibodies thereto, and genes
coding therefor, can be used as diagnostic reagents to
detect the presence of infection in a mammalian
subject. Similarly, the genes encoding the proteins
can be cloned and used to design probes to detect and
20   isolate homologous genes in other bacterial strains.
For example, fragments comprising at least about 15-20
nucleotides, more preferably at least about 20-50
nucleotides, and most preferably about 60-100 or more
nucleotides, will find use in these embodiments. The
25   *S. uberis* LF-binding proteins also find use in
purifying bovine LFs from streptococcal species and
from recombinant host cells expressing the same.

S. uberis Lf binding proteins can be used in
vaccine compositions either alone or in combination
30   with other bacterial, fungal, viral or protozoal
antigens. These antigens can be provided separately
or even as fusion proteins comprising one or more
epitopes of an LF-binding protein fused to one or more
of these antigens. For example, other immunogenic
35   proteins from *S. uberis*, such as the CAMP factor,
hyaluronic acid capsule, hyaluronidase, R-like protein

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and plasminogen activator, can be administered with
the LF-binding protein. Additionally, immunogenic
proteins from other organisms involved in mastitis,
such as from the genera *Staphylococcus*,

5   *Corynebacterium*, *Pseudomonas*, *Nocardia*, *Clostridium*,
Mycobacterium, *Mycoplasma*, *Pasteurella*, *Prototheca*,
other streptococci, coliform bacteria, as well as
yeast, can be administered along with the bLF-binding
proteins described herein to provide a broad spectrum

10   of protection. Thus, for example, immunogenic
proteins from *Staphylococcus aureus*, *Str. agalactiae*,
Str. dysgalactiae, *Str. zooepidemicus*, *Corynebacterium
pyogenes*, *Pseudomonas aeruginosa*, *Nocardia asteroides*,
Clostridium perfringens, *Escherichia coli*,

15   *Enterobacter aerogenes* and *Klebsiella spp.* can be
provided along with the bLF-binding proteins of the
present invention.

            Production of LF-Binding Protein
20   The above described LF-binding proteins and
active fragments, analogs and chimeric proteins
derived from the same, can be produced by a variety of
methods. Specifically, LF-binding proteins can be
isolated directly from bacteria which express the

25   same. This is generally accomplished by first
preparing a crude extract which lacks cellular
components and several extraneous proteins. The
desired proteins can then be further purified i.e. by
column chromatography, HPLC, immunoadsorbent

30   techniques or other conventional methods well known in
the art.
           Alternatively, the proteins can be
recombinantly produced as described herein. As
explained above, these recombinant products can take

35   the form of partial protein sequences, full-length
sequences, precursor forms that include signal

sequences, mature forms without signals, or even
fusion proteins (e.g., with an appropriate leader for
the recombinant host, or with another subunit antigen
sequence for *Streptococcus* or another pathogen).

5   The *lbp* genes of the present invention can
be isolated based on the ability of the protein
products to bind LF, using LF-binding assays as
described below. Thus, gene libraries can be
constructed and the resulting clones used to transform
10   an appropriate host cell. Colonies can be pooled and
screened for clones having LF-binding activity.
Colonies can also be screened using polyclonal serum
or monoclonal antibodies to the LF-binding protein.

          Alternatively, once the amino acid sequences
15   are determined, oligonucleotide probes which contain
the codons for a portion of the determined amino acid
sequences can be prepared and used to screen genomic
or cDNA libraries for genes encoding the subject
proteins. The basic strategies for preparing
20   oligonucleotide probes and DNA libraries, as well as
their screening by nucleic acid hybridization, are
well known to those of ordinary skill in the art.
See, e.g., *DNA Cloning*: Vol. I, *supra*; *Nucleic Acid
Hybridization*, *supra*; *Oligonucleotide Synthesis*,
25   *supra*; Sambrook et al., *supra*. Once a clone from the
screened library has been identified by positive
hybridization, it can be confirmed by restriction
enzyme analysis and DNA sequencing that the particular
library insert contains an LF-binding protein gene or
30   a homolog thereof. The genes can then be further
isolated using standard techniques and, if desired,
PCR approaches or restriction enzymes employed to
delete portions of the full-length sequence.

          Similarly, genes can be isolated directly
35   from bacteria using known techniques, such as phenol
extraction and the sequence further manipulated to

produce any desired alterations. See, e.g., Sambrook
et al., *supra*, for a description of techniques used to
obtain and isolate DNA.

5   Alternatively, DNA sequences encoding the
proteins of interest can be prepared synthetically
rather than cloned. The DNA sequences can be designed
with the appropriate codons for the particular amino
acid sequence. In general, one will select preferred
codons for the intended host if the sequence will be
10   used for expression. The complete sequence is
assembled from overlapping oligonucleotides prepared
by standard methods and assembled into a complete
coding sequence. See, e.g., Edge (1981) *Nature*
292:756; Nambair et al. (1984) *Science* 223:1299; Jay
15   et al. (1984) *J. Biol. Chem.* 259:6311.

          Once coding sequences for the desired
proteins have been prepared or isolated, they can be
cloned into any suitable vector or replicon. Numerous
cloning vectors are known to those of skill in the
20   art, and the selection of an appropriate cloning
vector is a matter of choice. Examples of recombinant
DNA vectors for cloning and host cells which they can
transform include the bacteriophage λ (*E. coli*),
pBR322 (*E. coli*), pACYC177 (*E. coli*), pKT230
25   (gram-negative bacteria), pGV1106 (gram-negative
bacteria), pLAFR1 (gram-negative bacteria), pME290
(non-*E. coli* gram-negative bacteria), pHV14 (*E. coli*
and *Bacillus subtilis*), pBD9 (*Bacillus*), pIJ61
(*Streptomyces*), pUC6 (*Streptomyces*), YIp5
30   (*Saccharomyces*), YCp19 (*Saccharomyces*) and bovine
papilloma virus (mammalian cells). See, Sambrook et
al., *supra*; *DNA Cloning*, *supra*; B. Perbal, *supra*.

          The gene can be placed under the control of
a promoter, ribosome binding site (for bacterial
35   expression) and, optionally, an operator (collectively
referred to herein as "control" elements), so that the

DNA sequence encoding the desired protein is
transcribed into RNA in the host cell transformed by a
vector containing this expression construction. The
coding sequence may or may not contain a signal

5   peptide or leader sequence. If a signal sequence is
included, it can either be the native, homologous
sequence, or a heterologous sequence. For example,
the signal sequence for *S. uberis* LF-binding protein
(shown in Figure 2A), can be used for secretion

10   thereof, as can a number of other signal sequences,
well known in the art. Leader sequences can be
removed by the host in post-translational processing.
See, e.g., U.S. Patent Nos. 4,431,739; 4,425,437;
4,338,397.

15   Other regulatory sequences may also be
desirable which allow for regulation of expression of
the protein sequences relative to the growth of the
host cell. Regulatory sequences are known to those of
skill in the art, and examples include those which

20   cause the expression of a gene to be turned on or off
in response to a chemical or physical stimulus,
including the presence of a regulatory compound.
Other types of regulatory elements may also be present
in the vector, for example, enhancer sequences.

25   The control sequences and other regulatory
sequences may be ligated to the coding sequence prior
to insertion into a vector, such as the cloning
vectors described above. Alternatively, the coding
sequence can be cloned directly into an expression

30   vector which already contains the control sequences
and an appropriate restriction site.

              In some cases it may be necessary to modify
the coding sequence so that it may be attached to the
control sequences with the appropriate orientation;

35   i.e., to maintain the proper reading frame. It may
also be desirable to produce mutants or analogs of the

LF-binding protein. Mutants or analogs may be
prepared by the deletion of a portion of the sequence
encoding the protein, by insertion of a sequence,
and/or by substitution of one or more nucleotides

5   within the sequence. Techniques for modifying
nucleotide sequences, such as site-directed
mutagenesis, are described in, e.g., Sambrook et al.,
supra; *DNA Cloning*, *supra*; *Nucleic Acid Hybridization*,
supra.

10   The expression vector is then used to
transform an appropriate host cell. A number of
mammalian cell lines are known in the art and include
immortalized cell lines available from the American
Type Culture Collection (ATCC), such as, but not

15   limited to, Chinese hamster ovary (CHO) cells, HeLa
cells, baby hamster kidney (BHK) cells, monkey kidney
cells (COS), human hepatocellular carcinoma cells
(e.g., Hep G2), Madin-Darby bovine kidney ("MDBK")
cells, as well as others. Similarly, bacterial hosts

20   such as *E. coli*, *Bacillus subtilis*, and *Streptococcus
spp.*, will find use with the present expression
constructs. Yeast hosts useful in the present
invention include *inter alia*, *Saccharomyces
cerevisiae*, *Candida albicans*, *Candida maltosa*,

25   *Hansenula polymorpha*, *Kluyveromyces fragilis*,
Kluyveromyces lactis, *Pichia guillerimondii*, *Pichia
pastoris*, *Schizosaccharomyces pombe* and *Yarrowia
lipolytica*. Insect cells for use with baculovirus
expression vectors include, *inter alia*, *Aedes aegypti*,

30   *Autographa californica*, *Bombyx mori*, *Drosophila
melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia
ni*.

Depending on the expression system and host
selected, the proteins of the present invention are

35   produced by culturing host cells transformed by an
expression vector described above under conditions

whereby the protein of interest is expressed. The
protein is then isolated from the host cells and puri-
fied. If the expression system secretes the protein
into the growth media, the protein can be purified

5   directly from the media. If the protein is not
secreted, it is isolated from cell lysates. The
selection of the appropriate growth conditions and
recovery methods are within the skill of the art.

     The proteins of the present invention may

10   also be produced by chemical synthesis such as solid
phase peptide synthesis, using known amino acid
sequences or amino acid sequences derived from the DNA
sequence of the genes of interest. Such methods are
known to those skilled in the art. See, e.g., J. M.

15   Stewart and J. D. Young, *Solid Phase Peptide
Synthesis*, 2nd Ed., Pierce Chemical Co., Rockford, IL
(1984) and G. Barany and R. B. Merrifield, *The
Peptides: Analysis, Synthesis, Biology*, editors E.
Gross and J. Meienhofer, Vol. 2, Academic Press, New

20   York, (1980), pp. 3-254, for solid phase peptide
synthesis techniques; and M. Bodansky, *Principles of
Peptide Synthesis*, Springer-Verlag, Berlin (1984) and
E. Gross and J. Meienhofer, Eds., *The Peptides:
Analysis, Synthesis, Biology*, *supra*, Vol. 1, for

25   classical solution synthesis. Chemical synthesis of
peptides may be preferable if a small fragment of the
antigen in question is capable of raising an
immunological response in the subject of interest.

     The LF-binding proteins of the present

30   invention, or their fragments, can be used to produce
antibodies, both polyclonal and monoclonal. If
polyclonal antibodies are desired, a selected mammal,
(e.g., mouse, rabbit, goat, horse, etc.) is immunized
with an antigen of the present invention, or its

35   fragment, or a mutated antigen. Serum from the
immunized animal is collected and treated according to

known procedures. See, e.g., Jurgens et al. (1985) *J. Chrom.* 348:363-370. If serum containing polyclonal antibodies is used, the polyclonal antibodies can be purified by immunoaffinity chromatography, using known

5   procedures.

Monoclonal antibodies to the LF-binding proteins and to the fragments thereof, can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies

10   by using hybridoma technology is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M.

15   Schreier et al., *Hybridoma Techniques* (1980); Hammerling *et al.*, *Monoclonal Antibodies and T-cell Hybridomas* (1981); Kennett et al., *Monoclonal Antibodies* (1980); see also U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,452,570;

20   4,466,917; 4,472,500, 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against the LF-binding protein, or fragment thereof, can be screened for various properties; i.e., for isotype, epitope, affinity, etc. Monoclonal antibodies are

25   useful in purification, using immunoaffinity techniques, of the individual antigens which they are directed against. Both polyclonal and monoclonal antibodies can also be used for passive immunization or can be combined with subunit vaccine preparations

30   to enhance the immune response. Polyclonal and monoclonal antibodies are also useful for diagnostic purposes.

Vaccine Formulations and Administration

35   The LF-binding proteins of the present invention can be formulated into vaccine compositions,

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either alone or in combination with other antigens,
for use in immunizing subjects as described below.
Methods of preparing such formulations are described
in, e.g., *Remington's Pharmaceutical Sciences*, Mack

5   Publishing Company, Easton, Pennsylvania, 18 Edition,
1990. Typically, the vaccines of the present
invention are prepared as injectables, either as
liquid solutions or suspensions. Solid forms suitable
for solution in or suspension in liquid vehicles prior

10   to injection may also be prepared. The preparation
may also be emulsified or the active ingredient
encapsulated in liposome vehicles. The active
immunogenic ingredient is generally mixed with a
compatible pharmaceutical vehicle, such as, for

15   example, water, saline, dextrose, glycerol, ethanol,
or the like, and combinations thereof. In addition,
if desired, the vehicle may contain minor amounts of
auxiliary substances such as wetting or emulsifying
agents and pH buffering agents.

20   Adjuvants which enhance the effectiveness of
the vaccine may also be added to the formulation.
Adjuvants may include for example, muramyl dipeptides,
avridine, aluminum hydroxide, dimethyldioctadecyl
ammonium bromide (DDA), oils, oil-in-water emulsions,

25   saponins, cytokines, and other substances known in the
art.
         The Lf binding protein may be linked to a
carrier in order to increase the immunogenicity
thereof. Suitable carriers include large, slowly

30   metabolized macromolecules such as proteins, including
serum albumins, keyhole limpet hemocyanin,
immunoglobulin molecules, thyroglobulin, ovalbumin,
and other proteins well known to those skilled in the
art; polysaccharides, such as sepharose, agarose,

35   cellulose, cellulose beads and the like; polymeric
amino acids such as polyglutamic acid, polylysine, and

the like; amino acid copolymers; and inactive virus
particles.

The LF-binding proteins may be used in their
native form or their functional group content may be
5   modified by, for example, succinylation of lysine
residues or reaction with Cys-thiolactone. A
sulfhydryl group may also be incorporated into the
carrier (or antigen) by, for example, reaction of
amino functions with 2-iminothiolane or the
10   N-hydroxysuccinimide ester of 3-(4-dithiopyridyl)
propionate. Suitable carriers may also be modified to
incorporate spacer arms (such as hexamethylene diamine
or other bifunctional molecules of similar size) for
attachment of peptides.

15   Other suitable carriers for the LF-binding
proteins of the present invention include VP6
polypeptides of rotaviruses, or functional fragments
thereof, as disclosed in U.S. Patent No. 5,071,651.
Also useful is a fusion product of a viral protein and
20   the subject immunogens made by methods disclosed in
U.S. Patent No. 4,722,840. Still other suitable
carriers include cells, such as lymphocytes, since
presentation in this form mimics the natural mode of
presentation in the subject, which gives rise to the
25   immunized state. Alternatively, the proteins of the
present invention may be coupled to erythrocytes,
preferably the subject's own erythrocytes. Methods of
coupling peptides to proteins or cells are known to
those of skill in the art.

30   Furthermore, the LF-binding proteins (or
complexes thereof) may be formulated into vaccine
compositions in either neutral or salt forms.
Pharmaceutically acceptable salts include the acid
addition salts (formed with the free amino groups of
35   the active polypeptides) and which are formed with in-
organic acids such as, for example, hydrochloric or

phosphoric acids, or such organic acids as acetic,
oxalic, tartaric, mandelic, and the like. Salts
formed from free carboxyl groups may also be derived
from inorganic bases such as, for example, sodium,
5   potassium, ammonium, calcium, or ferric hydroxides,
and such organic bases as isopropylamine,
trimethylamine, 2-ethylamino ethanol, histidine,
procaine, and the like.

     Vaccine formulations will contain a
10   "therapeutically effective amount" of the active
ingredient, that is, an amount capable of eliciting an
immune response in a subject to which the composition
is administered. In the treatment and prevention of
mastitis, for example, a "therapeutically effective
15   amount" would preferably be an amount that enhances
resistance of the mammary gland to new infection
and/or reduces the clinical severity of the disease.
Such protection will be demonstrated by either a
reduction or lack of symptoms normally displayed by an
20   infected host, a quicker recovery time and/or a
lowered somatic cell count in milk from the infected
quarter. For example, the ability of the composition
to retain or bring the somatic cell count (SCC) in
milk below about 500,000 cells per ml, the threshold
25   value set by the International Dairy Federation, above
which, animals are considered to have clinical
mastitis, will be indicative of a therapeutic effect.

     The exact amount is readily determined by
one skilled in the art using standard tests. The LF-
30   binding protein concentration will typically range
from about 1% to about 95% (w/w) of the composition,
or even higher or lower if appropriate. With the
present vaccine formulations, 20 to 500 µg of active
ingredient per ml of injected solution should be
35   adequate to raise an immunological response when a
dose of 1 to 3 ml per animal is administered.

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To immunize a subject, the vaccine is
generally administered parenterally, usually by
intramuscular injection. Other modes of
administration, however, such as subcutaneous,
5   intraperitoneal and intravenous injection, are also
acceptable. The quantity to be administered depends
on the animal to be treated, the capacity of the
animal's immune system to synthesize antibodies, and
the degree of protection desired. Effective dosages
10 can be readily established by one of ordinary skill in
the art through routine trials establishing dose
response curves. The subject is immunized by
administration of the vaccine in at least one dose,
and preferably two doses. Moreover, the animal may be
15 administered as many doses as is required to maintain
a state of immunity to infection.

Additional vaccine formulations which are
suitable for other modes of administration include
suppositories and, in some cases, aerosol, intranasal,
20 oral formulations, and sustained release formulations.
For suppositories, the vehicle composition will
include traditional binders and carriers, such as,
polyalkaline glycols, or triglycerides. Such
suppositories may be formed from mixtures containing
25 the active ingredient in the range of about 0.5% to
about 10% (w/w), preferably about 1% to about 2%.
Oral vehicles include such normally employed
excipients as, for example, pharmaceutical grades of
mannitol, lactose, starch, magnesium, stearate, sodium
30 saccharin cellulose, magnesium carbonate, and the
like. These oral vaccine compositions may be taken in
the form of solutions, suspensions, tablets, pills,
capsules, sustained release formulations, or powders,
and contain from about 10% to about 95% of the active
35 ingredient, preferably about 25% to about 70%.

Intranasal formulations will usually include vehicles that neither cause irritation to the nasal mucosa nor significantly disturb ciliary function. Diluents such as water, aqueous saline or other known

5   substances can be employed with the subject invention. The nasal formulations may also contain preservatives such as, but not limited to, chlorobutanol and benzalkonium chloride. A surfactant may be present to enhance absorption of the subject proteins by the

10   nasal mucosa.

Controlled or sustained release formulations are made by incorporating the protein into carriers or vehicles such as liposomes, nonresorbable impermeable polymers such as ethylenevinyl acetate copolymers and

15   Hytrel® copolymers, swellable polymers such as hydrogels, or resorbable polymers such as collagen and certain polyacids or polyesters such as those used to make resorbable sutures. The LF-binding proteins can also be delivered using implanted mini-pumps, well

20   known in the art.

The LF-binding proteins of the instant invention can also be administered via a carrier virus which expresses the same. Carrier viruses which will find use with the instant invention include but are

25   not limited to the vaccinia and other pox viruses, adenovirus, and herpes virus. By way of example, vaccinia virus recombinants expressing the novel proteins can be constructed as follows. The DNA encoding the particular protein is first inserted into

30   an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence encoding thymidine kinase (TK). This vector is then used to transfect cells which are simultaneously infected with vaccinia. Homologous

35   recombination serves to insert the vaccinia promoter plus the gene encoding the instant protein into the

viral genome. The resulting TK⁻recombinant can be
selected by culturing the cells in the presence of 5-
bromodeoxyuridine and picking viral plaques resistant
thereto.

5   An alternative route of administration
involves gene therapy or nucleic acid immunization.
Thus, nucleotide sequences (and accompanying
regulatory elements) encoding the subject LF-binding
proteins can be administered directly to a subject for
10   *in vivo* translation thereof. Alternatively, gene
transfer can be accomplished by transfecting the
subject's cells or tissues *ex vivo* and reintroducing
the transformed material into the host. DNA can be
directly introduced into the host organism, i.e., by
15   injection (see International Publication No.
WO/90/11092; and Wolff et al. (1990) *Science* <u>247</u>:1465-
1468). Liposome-mediated gene transfer can also be
accomplished using known methods. See, e.g., Hazinski
et al. (1991) *Am. J. Respir. Cell Mol. Biol.* <u>4</u>:206-
20   209; Brigham et al. (1989) *Am. J. Med. Sci.* <u>298</u>:278-
281; Canonico et al. (1991) *Clin. Res.* <u>39</u>:219A; and
Nabel et al. (1990) *Science* <u>249</u>:1285-1288. Targeting
agents, such as antibodies directed against surface
antigens expressed on specific cell types, can be
25   covalently conjugated to the liposomal surface so that
the nucleic acid can be delivered to specific tissues
and cells susceptible to infection.

                      <u>Diagnostic Assays</u>
30   As explained above, the LF-binding proteins
of the present invention may also be used as
diagnostics to detect the presence of reactive
antibodies of *S. uberis* in a biological sample in
order to determine the presence of *S. uberis*
35   infection. For example, the presence of antibodies
reactive with LF-binding proteins can be detected

using standard electrophoretic and immunodiagnostic
techniques, including immunoassays such as
competition, direct reaction, or sandwich type assays.
Such assays include, but are not limited to, Western

5   blots; agglutination tests; enzyme-labeled and
mediated immunoassays, such as ELISAs; biotin/avidin
type assays; radioimmunoassays; immunoelectrophoresis;
immunoprecipitation, etc. The reactions generally
include revealing labels such as fluorescent,

10 chemiluminescent, radioactive, enzymatic labels or dye
molecules, or other methods for detecting the
formation of a complex between the antigen and the
antibody or antibodies reacted therewith.

          The aforementioned assays generally involve

15 separation of unbound antibody in a liquid phase from
a solid phase support to which antigen-antibody
complexes are bound. Solid supports which can be used
in the practice of the invention include substrates
such as nitrocellulose (e.g., in membrane or

20 microtiter well form); polyvinylchloride (e.g., sheets
or microtiter wells); polystyrene latex (e.g., beads
or microtiter plates); polyvinylidine fluoride;
diazotized paper; nylon membranes; activated beads,
magnetically responsive beads, and the like.

25   Typically, a solid support is first reacted
with a solid phase component (e.g., one or more LF-
binding proteins) under suitable binding conditions
such that the component is sufficiently immobilized to
the support. Sometimes, immobilization of the antigen

30 to the support can be enhanced by first coupling the
antigen to a protein with better binding properties.
Suitable coupling proteins include, but are not
limited to, macromolecules such as serum albumins
including bovine serum albumin (BSA), keyhole limpet

35 hemocyanin, immunoglobulin molecules, thyroglobulin,
ovalbumin, and other proteins well known to those

skilled in the art. Other molecules that can be used
to bind the antigens to the support include
polysaccharides, polylactic acids, polyglycolic acids,
polymeric amino acids, amino acid copolymers, and the
5   like. Such molecules and methods of coupling these
molecules to the antigens, are well known to those of
ordinary skill in the art. See, e.g., Brinkley, M.A.
Bioconjugate Chem. (1992) 3:2-13; Hashida et al., *J.
Appl. Biochem.* (1984) 6:56-63; and Anjaneyulu and
10   Staros, *International J. of Peptide and Protein Res.*
(1987) 30:117-124.

         After reacting the solid support with the
solid phase component, any non-immobilized solid-phase
components are removed from the support by washing,
15   and the support-bound component is then contacted with
a biological sample suspected of containing ligand
moieties (e.g., antibodies toward the immobilized
antigens) under suitable binding conditions. After
washing to remove any non-bound ligand, a secondary
20   binder moiety is added under suitable binding
conditions, wherein the secondary binder is capable of
associating selectively with the bound ligand. The
presence of the secondary binder can then be detected
using techniques well known in the art.

25   More particularly, an ELISA method can be
used, wherein the wells of a microtiter plate are
coated with an LF-binding protein. A biological
sample containing or suspected of containing anti-LF-
binding protein immunoglobulin molecules is then added
30   to the coated wells. After a period of incubation
sufficient to allow antibody binding to the
immobilized antigen, the plate(s) can be washed to
remove unbound moieties and a detectably labeled
secondary binding molecule added. The secondary
35   binding molecule is allowed to react with any captured
sample antibodies, the plate washed and the presence

of the secondary binding molecule detected using
methods well known in the art.

      Thus, in one particular embodiment, the
presence of bound anti-LF-binding antigen ligands from
a biological sample can be readily detected using a
secondary binder comprising an antibody directed
against the antibody ligands. A number of anti-bovine
immunoglobulin (Ig) molecules are known in the art
which can be readily conjugated to a detectable enzyme
label, such as horseradish peroxidase, alkaline
phosphatase or urease, using methods known to those of
skill in the art. An appropriate enzyme substrate is
then used to generate a detectable signal. In other
related embodiments, competitive-type ELISA techniques
can be practiced using methods known to those skilled
in the art.

      Assays can also be conducted in solution,
such that the LF-binding proteins and antibodies
specific for those proteins form complexes under
precipitating conditions. In one particular
embodiment, LF-binding proteins can be attached to a
solid phase particle (e.g., an agarose bead or the
like) using coupling techniques known in the art, such
as by direct chemical or indirect coupling. The
antigen-coated particle is then contacted under
suitable binding conditions with a biological sample
suspected of containing antibodies for the LF-binding
proteins. Cross-linking between bound antibodies
causes the formation of particle-antigen-antibody
complex aggregates which can be precipitated and
separated from the sample using washing and/or
centrifugation. The reaction mixture can be analyzed
to determine the presence or absence of antibody-
antigen complexes using any of a number of standard
methods, such as those immunodiagnostic methods
described above.

In yet a further embodiment, an
immunoaffinity matrix can be provided, wherein a
polyclonal population of antibodies from a biological
sample suspected of containing anti-LF-binding

5   molecules is immobilized to a substrate. In this
regard, an initial affinity purification of the sample
can be carried out using immobilized antigens. The
resultant sample preparation will thus only contain
anti-*S. uberis* moieties, avoiding potential

10   nonspecific binding properties in the affinity
support. A number of methods of immobilizing
immunoglobulins (either intact or in specific
fragments) at high yield and good retention of antigen
binding activity are known in the art. Not being

15   limited by any particular method, immobilized protein
A or protein G can be used to immobilize
immunoglobulins.

Accordingly, once the immunoglobulin
molecules have been immobilized to provide an

20   immunoaffinity matrix, labeled LF-binding proteins are
contacted with the bound antibodies under suitable
binding conditions. After any non-specifically bound
antigen has been washed from the immunoaffinity
support, the presence of bound antigen can be

25   determined by assaying for label using methods known
in the art.

Additionally, antibodies raised to the LF-
binding proteins, rather than the LF-binding proteins
themselves, can be used in the above-described assays

30   in order to detect the presence of antibodies to the
proteins in a given sample. These assays are
performed essentially as described above and are well
known to those of skill in the art.

The above-described assay reagents,

35   including the LF-binding proteins, or antibodies
thereto, can be provided in kits, with suitable

instructions and other necessary reagents, in order to
conduct immunoassays as described above. The kit can
also contain, depending on the particular immunoassay
used, suitable labels and other packaged reagents and
5   materials (i.e. wash buffers and the like). Standard
immunoassays, such as those described above, can be
conducted using these kits.·

        Below are examples of specific embodiments
for carrying out the present invention. The examples
10 are offered for illustrative purposes only, and are
not intended to limit the scope of the present
invention in any way.

C.   Experimental

15

Example 1
Identification of a Bovine Lactoferrin-Binding Protein in *S. Uberis*

20   Materials and Methods
Bacterial strains and cultural conditions.

        S. uberis strain (su-1) from the American
Type Culture Collection (ATCC 9927) was used for
study. Bacteria were grown on base #2 sheep blood agar
25 plates (PML Microbiologicals) at 37°C for 18 h. Iron-
restricted conditions were achieved in Todd-Hewitt
broth supplemented with 0.3% yeast extract (THB-YE) by
the addition of 800 μM EDDA, 800 μM dipyridyl or 100
μM desferrioxamine mesylate. All of the iron
30 chelators were obtained from Sigma.

Preparation of bacterial cell wall.

        Cell wall components of *S. uberis* were
extracted as described by Baker et al. (1976) *J. Exp.*
35 *Med.* 143:258-270. Twenty base #2 blood agar plates
were inoculated with *S. uberis* and incubated for 18 h

at 37°C. Bacterial cells were collected, washed once
with 200 ml 0.85% saline and resuspended in 50 ml of
extraction buffer (0.05 M Na_2HPO_4, 0.15 M NaCl, 0.01 M
EDTA, pH 7.4). Cell walls were extracted by shaking
5   with glass beads (4-mm diameter) for 20 h at 37°C.
After centrifugation at 48,300 g for 20 min,
supernatant (cell wall extract) was collected,
filtered (0.22 μM Nalgene filter), dialysed against
distilled water, lyophilized and resuspended in 1 ml
10 of distilled water.

Preparation of iron-binding proteins.
        All iron-binding proteins, including bLf
(from bovine milk), bovine transferrin (bTf), human
15 lactoferrin (hLf) and human transferrin (hTf), were
purchased from Sigma in the most iron-free form
available. Iron-saturated proteins and the
apoproteins were prepared by methods described
previously (Mazurier and Spik (1980) *Biochim. Biophys.*
20 *Acta* 629:399-408).

Preparation of ^{125}I-labelled bLF.
        Bovine Lf was iodinated by the
lactoperoxidase method of Thorell and Johansson (1971)
25 *Biochim. Biophys. Acta* 251:363-368. Approximately 70
μg of bLf (33% iron-saturated) was used for
iodination; ^{125}I-labelled protein was separated from
free Na[125]I by chromatography on a Sephadex G-25
column. The labelled protein was aliquoted and stored
30 at -70°C until use. Lactoperoxidase was purchased
from Boehringer-Mannheim; and Na[125]I from Amersham.

Lactoferrin-binding assays.
        The binding assays were performed as
35 described (Naidu et al. (1990) *J. Clin. Microbiol.*
28:2312-2319; Naidu et al. (1991) *J. Med. Micrbiol.*

<u>34</u>:323-328; Naidu et al. (1991) *J. Dairy Sci.* <u>74</u>:1218-
1226; Naidu et al. (1992) *J. Med. Microbiol.* <u>36</u>:177-
183). Bacterial cells were harvested from culture
media, washed once in 0.1 M phosphate-buffered saline

5   (PBS), pH 7.2, and resuspended in PBS containing 1%
bovine serum albumin (PBS-1% BSA) to a density of 10^{10}
bacteria/ml (c. $OD_{600}=1.5$). To determine saturation
time, 10^9 bacteria (in 0.1 ml of PBS-1% BSA) were mixed
and incubated with 0.1 ml of ^{125}I-bLf solution (6.9 nM

10 in PBS-1 % BSA) for periods of 5, 10, 15, 20, 25, 30,
60, 90, 120, 150 min at room temperature. Bacteria
were pelleted and washed three times with 1 ml of ice-
cold PBS containing 0.1% Tween 20. Radioactivity
bound to the bacterial pellet was measured in a γ-

15 counter. In competitive binding experiments, 10^9
bacteria were mixed with 2×10^5 cpm ^{125}I-bLf in the
presence of serially-diluted unlabelled bLf and
incubated at room temperature for 2 h. Total input,
cell bound and free proteins were calculated and

20 subjected to Scatchard analysis (Scatchard, G. (1949)
Ann. N. Y. Acad. Sci. <u>51</u>:660-672). When evaluating
the inhibitory effect of bLf, apo-bLf, bTf, hLF and
hTf on ^{125}I-bLf binding, the unlabelled proteins were
used at concentration of 5.5 μM. All samples were

25 tested in triplicate, and each experiment was repeated
at least twice. The data presented are the means of
two independent experiments (unless otherwise stated).

<u>Proteolytic and heat treatment of *S. uberis*.</u>

30   Bacteria (1 ml containing 10^{10} cells) were
treated with proteases at 37°C for 2 h. Trypsin
(Sigma) hydrolysis was performed in 100 mM Tris-HCl
(pH 8.0), with a final enzyme concentration of 2,500
u/ml, and the reaction was stopped by addition of

35 phenylmethylsulfonyl fluoride (500 $\mu g/ml$). Pepsin
(Sigma) digestion was performed in 100 mM sodium

acetate buffer (pH 4.5), with an enzyme concentration
of 1,000 u/ml, and the pH of the reaction mixture was
raised to 7.4 to stop the hydrolysis. Proteinase K
(Boehringer mannheim) treatment was carried out in 40

5   mM potassium phosphate buffer (pH 7.5), and the
digestion was inhibited by the addition of
phenylmethylsulfonyl fluoride (500 μg/ml). For heat
treatment, the bacterial suspension (10^{10} cell/ml) was
incubated in a water bath for 1 h at each of the

10   following temperatures: 50°C, 80°C and 100°C. Both
enzyme and heat-treated cells were washed once in PBS
and resuspended in PBS-1% BSA prior to the binding
experiments.

15   PAGE and Western blotting.
             SDS-polyacrylamide gel electrophoresis
(PAGE) of proteins was performed using the method
described by Laemmli (Laemmli, U.K. (1970) Nature
227:680-685). Samples were solubilized in sample

20   buffer at 37°C for 30 min in the absence of 2-
mercaptoethanol (non-reducing conditions) or at 100°C
for 5 min in the presence of 1% 2-mercaptoethanol
(reducing conditions). Proteins were
electrophoretically transferred to nitrocellulose

25   membranes as recommended by the supplier (Bio-Rad) and
blocked with TBS-1% BSA.
             To identify the putative bLf-binding
protein, Western blots were probed with ^{125}I-bLf as
follows. ^{125}I-bLf was added to the membrane to a final

30   concentration of 80 ng/ml in TBS-1% BSA and incubated
at room temperature for 2 h. After three washes with
TBS containing 0.05% Tween 20, the membrane was
exposed to X-ray film for 24 h at room temperature.
To compete the ^{125}I-bLf binding, the transferred

35   membrane was incubated with 35 μg/ml of unlabelled bLf
for 2 h before incubation with ^{125}I-bLf.

Results

Time-dependent binding of bLF to *S. uberis*.

S. uberis strain su-1 was tested for bLf
binding in a ^{125}I-labelled protein binding assay. To

5   study the kinetics of ^{125}I-bLf binding with *S. uberis*,
binding was measured at different time intervals
(Figure 7). The time course showed that ^{125}I-bLf could
bind to *S. uberis* in a time-dependent manner, with a
requirement of approximately 90 min for 100%

10   saturation. This binding saturation time was the
basis for determining the incubation time in later
binding experiments.

BLf receptor saturability, affinity and copy number.

15   A competitive binding experiment using bLf
(33% iron-saturated) as both radioligand and
competitor was performed, and the specificity of bLf
binding by *S. uberis* was demonstrated (Figure 8).
Unlabelled bLf effectively displaced the binding of

20   ^{125}I-bLf to *S. uberis* in a dose-dependent manner. A
concentration of approximately 270 nM of unlabelled
bLf caused 50% blocking of ^{125}I-bLf uptake (indicated
by dotted lines). Scatchard plot analysis showed
linearity, thus the demonstration of one bLf binding

25   component is expected. The number of bLf molecules
bound per *S. uberis* cell calculated from Scatchard
plot was approximately 7800, with an affinity (Kd) of
1.0×10^{-7}M.

To determine whether bLf iron saturation

30   could influence receptor binding, apo-bLf was used as
a competitor in the ^{125}I-bLf binding assay. The
results showed that apo-bLf could inhibit ^{125}I-bLF-
binding as effectively as the iron-saturated bLf
(Table 1), indicating that both apo-bLf and iron-

35   saturated bLf had the same binding receptor on the *S.
uberis* cell.

To examine further the specificity of
binding, the abilities of bTf, hLf and hTf to inhibit
the binding of ^{125}I-bLf to *S. uberis* cells were also
evaluated (Table 1). None of these proteins

5   interfered with the binding of ^{125}I-bLf to *S. uberis*,
suggesting that the binding was bLf-specific.

Influence of iron-restricted conditions on bLf
binding.

10   In an attempt to determine if the bLf-
binding property of *S. uberis* was mediated by an iron-
regulated bacterial component, EDDA, dipyridyl or
desferrioxamine mesylate were incorporated in THB-YE
broth to reduce the availability of iron. Cells from

15   these iron-restricted conditions did not show higher
^{125}I-bLf binding than those from normal cultural
condition (Figure 9), indicating that iron-restricted
condition did not notably modify the saturation of *S.
uberis* by bLf.

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Table 1. Inhibitory effects of unlabelled proteins and enzyme or heat treatment of bacteria on ^{125}I-bLf binding to *S. uberis*.

Inhibitor or cell treatment	Percentage inhibition[1] or decrease[2] in binding	
	mean	SD
Inhibitor		
bLf	94.6	0.1
apo-bLf	94.7	0.2
bTf	2.5	1.4
hLf	-14.0	2.4
hTf	1.9	1.3
Proteases		
Pepsin	85.7	3.7
Trypsin	89.1	4.4
Proteinase K	92.7	2.4
Heating		
50°C	-4.3	6.0
80°C	29.6	2.1
100°C	65.6	4.8

[1] Inhibition values were calculated as relative percentage of bLf binding to bacteria suspended in PBS in the absence of any inhibitor.
[2] Decrease in binding were calculated as relative percentage of bLf binding to bacteria without any treatment.

<u>Sensitivity of the bLf binding component to protease hydrolysis and heat treatment.</u>

Pepsin, trypsin and proteinase k treatment of *S. uberis* cells could abolish bLf-binding (Table

1), indicating the involvement of a surface-exposed cell wall protein(s) in the binding. This proteinaceous component was susceptible to temperature, since heat treatment of bacteria reduced
5   binding to a certain degree (Table 1).

Identification of a cell wall bLf-binding protein.

The presence of the functionally active bLf-binding protein (Lbp) in the cell wall preparation of
10   S. uberis was detected by a Western blot probed with ^{125}I-bLf. Under non-reducing conditions, two components with apparent molecular weights of 165 and 76 kDa, respectively, were identified as bLf-binding proteins of S. uberis. Proteins under reducing
15   conditions lost the bLf binding activity to a great extent. The protein bands were demonstrated to be from specific binding to ^{125}I-bLf, since the presence of unlabelled bLf effectively blocked the binding.

20   Discussion

To demonstrate whether S. uberis was able to express a specific receptor for bLf, the inventors herein sought to determine whether the major prerequisites for a biological receptor could be
25   fulfilled, namely, ligand specificity and concentration-dependent saturability. Bovine Lf binding to S. uberis was time and concentration dependent (demonstrated by the ability of the unlabelled ligand to compete for binding with ^{125}I-
30   bLf), indicating the existence of a limited number of binding receptors on the cell surface. Scatchard plot analysis estimated that there were 7800 bLf-binding sites/cell. The affinity of the streptococcal receptor for bLf (1.0×10^{-7} M) is slightly lower than
35   that described by Naidu et al. (1991) J. Dairy Sci.

$\underline{74}$:1218-1226 for the bLf receptor of *S. aureus* (7.1 x 10^{-8} M).

    S. uberis did not distinguish between bovine apo-Lf and iron-saturated Lf, since both forms were equally effective at blocking ^{125}I-bLf binding in the competitive binding assay. This finding is similar to that described for the *N. meningitidis* Lf receptor (Schryvers and Morris (1988) *Infect. Immun.* $\underline{56}$:1144-1149) but contrasts with that for the Tf receptors.

    A common feature which has emerged from studies of the transferrin-binding proteins of Gram-negative bacteria is their remarkable specificity for the transferrin of their natural host. In competition binding assays, the streptococcal receptor described here also demonstrated some lactoferrin species specificity in that human Lf could not effectively block the binding of bovine Lf. Interestingly, *S. aureus* Lf receptors have been shown to bind lactoferrins from both human and bovine sources (Naidu et al. (1990) *J. Clin. Microbiol.* $\underline{28}$:2312-2319; Naidu et al. (1991) *J. Med. Micrbiol.* $\underline{34}$:323-328; Naidu et al. (1991) *J. Dairy Sci.* $\underline{74}$:1218-1226; Naidu et al. (1992) *J. Med. Microbiol.* $\underline{36}$:177-183). In this context, given that *S. uberis* is exclusively a bovine pathogen, while *S. aureus* causes infection in both humans and bovine, it is possible that the specificity for lactoferrins may also contribute to the host specificity of these bacteria. In addition, neither bovine nor human transferrin was able to block binding of ^{125}I-labelled bovine lactoferrin to the lactoferrin receptor of *S. uberis*. This observation is similar to that reported by Naidu et al. (1991) *J. Dairy Sci.* $\underline{74}$:1218-1226; Naidu et al. (1992) *J. Med. Microbiol.* $\underline{36}$:177-183, in which human or bovine lactoferrin-binding to lactoferrin receptors of *S. aureus* could not be blocked by human or bovine transferrin.

Although the expression of most of the
transferrin and lactoferrin receptors of Gram-negative
bacteria such as *N. meningitidis* and *H. influenza* is
iron regulated, *S. uberis* receptor activity was not

5   regulated by growth medium iron availability. The
results are consistent with the findings of Modun et
al. (1994) *Infect. Immun.* 62:3850-3858 and Rainard, P.
(1992) *FEMS Microbiol. Lett.* 98:235-240 who showed
that the binding of *S. aureus* to Tf and *S. agalactiae*

10   to Lf was not regulated by iron.

Having demonstrated the existence of bovine
lactoferrin-receptor on *S. uberis*, the inventors
herein sought to identify the bacterial cell wall
component(s) involved. Treatment of *S. uberis* cells

15   with heat and proteolytic enzymes abolished
lactoferrin-binding, indicating the probable
involvement of a protein(s) on the cell surface. A
cell wall extract of *S. uberis* was prepared and
solubilized in SDS-PAGE sample buffer under non-

20   reducing condition. Two lactoferrin-binding proteins
with molecular weights of 165 kDa and 76 kDa were
identified by ^{125}I-bLf binding after SDS-PAGE and
transfer to nitrocellulose. Since Scatchard plot
analysis indicated that *S. uberis* bears only one bLf

25   binding component, the 165 kDa protein is likely the
dimer form of the 76 kDa.

The lactoferrin-binding protein of *S. uberis*
differs from the transferrin receptors of *Haemophilus*
and *Neisseria* spp., which consist of two distinct

30   transferrin-binding proteins, termed Tbp1 and Tbp2,
which range in molecular weight from 68 to 105 kDa
depending on the strain. The *S. uberis* Lbp is also
different from the lactoferrin receptors of the
mentioned two species. To date, only one lactoferrin-

35   binding protein with a molecular weight of 98 to 105
kDa has been identified from those species above and

the gonococcal lactoferrin protein (Lbp1) has been
shown to share features with the functionally related
gonococcal Tbp1 (Biswas and Sparling, 1995). The
bovine lactoferrin receptor of *S. aureus* consists of
two distinct bLf-binding proteins with estimated
molecular weights of 92 and 67 kDa (Naidu et al.
(1991) *J. Dairy Sci.* <u>74</u>:1218-1226) and therefore
appears to be different than the receptor described
herein. Also, the streptococcal Lbp described here
appears to be different from the *S. aureus* human
lactoferrin-binding protein which is as an
approximately 450 kDa protein which under reducing
SDS-PAGE gel conditions resolves into two components
of 67 and 62 kDa.

<u>Example 2</u>
<u>Cloning and Characterization of *S. uberis* Lbp Gene</u>
<u>and its Upstream Mga</u>

<u>Materials and Methods</u>
<u>Bacterial strains, plasmids and media.</u>
        S. uberis strains used are listed in Table 2
below. Bacteria were grown on base #2 sheep blood
agar plates (PML Microbiologicals) at 37°C for 18 h,
or in Todd-Hewitt broth supplemented with 0.3% yeast
extract (THB-YE) at 37°C overnight. *E. coli* cells
were grown in Luria broth or on Luria broth-agar
plates. Ampicillin was used at 50 μg/ml for the
growth of *E. coli* strains containing recombinant
plasmids. The cloning vector used was pTZ18R (Mead et
al. (1986) *Protein Eng.* <u>1</u>:67-74).

<u>Preparation of bacterial cell wall, cell membranes,</u>
<u>periplasma, whole cell lysate and culture supernatant.</u>
        Cell wall components of *S. uberis* were
extracted as described in Example 1. Outer and inner

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membranes were isolated from *E. coli* cells by sucrose
density gradient centrifugation. *E. coli* periplasmic
proteins were prepared by the cold osmotic shock
method.

5   To prepare whole cell lysates of *E. coli*
transformants, bacteria were grown overnight,
collected, washed once in 0.1 M phosphate-buffered
saline (PBS), pH 7.2, resuspended in water, exposed to
one freeze-thaw cycle and sonicated for 2 min. After

10   centrifugation at 6000 x g for 20 min, the supernatant
(whole cell lysate) was collected, lyophilized and
resuspended in 1/100 culture volume of water.

          The 100 fold-concentrated culture
supernatant of the recombinant *E. coli* was obtained by

15   precipitation with 10% trichloroacetic acid (TCA).

Preparation of iron-saturated bLf and ^{125}I-labelled
bLf.

          33% iron-saturated bovine lactoferrin was

20   prepared and iodinated as described in Example 1.

Lactoferrin-binding assay.

          Bacterial cells were harvested from culture
media, washed once in 0.1 M PBS (pH 7.2), and

25   resuspended in PBS containing 1% bovine serum albumin
(PBS-1% BSA) to a density of 10^{10} bacteria/ml (c.
$OD_{600}=1.5$). To assess the bLf-binding ability of the
recombinant protein, increasing amounts of *E. coli*
cell lysate plus supernatant were mixed with

30   approximately 10^9 *S. uberis* and incubated with 1.0 x
10^4 cpm ^{125}I-bLf in a total volume of 200 μl. After 2
hours of incubation at room temperature, bacterial
cells were pelleted and washed three times with 1 ml
of ice-cold PBS containing 0.1 % Tween 20.

35   Radioactivity present in the bacterial pellet was
measured in a gamma-counter. All samples were tested

in triplicate, and the experiment was repeated twice.
The data presented are the mean of two independent
experiments ± standard deviation.

5   Preparation of antiserum.

          Serum against the recombinant Lbp of S.
uberis was raised in rabbits by subcutaneous injection
of 0.5 ml of TCA-precipitated culture supernatant of
recombinant E. coli in complete Freund's adjuvant.
10   Two subcutaneous boosts with the same amount of sample
in incomplete Freund's adjuvant were given to each
animal.

PAGE and Western blotting.
15   SDS-polyacrylamide gel electrophoresis
(PAGE) of proteins was performed as described by
Laemmli (Laemmli, U.K. (1970) Nature 227:680-685).
Samples under reducing and non-reducing conditions
were prepared as described in Example 1. Samples
20   dissolved in sample buffer with 3 M urea were boiled
for 30 min prior to loading. ^{125}I-bLf probed-Western
blotting was performed as in Example 1.
Immunoblotting probed with rabbit antiserum against
the recombinant Lbp was performed as follows.
25   Proteins were electroblotted onto nitrocellulose
membranes as recommended by the supplier (Bio-Rad).
Nonspecific binding was blocked by incubation in TBS
(10 mM Tris-HCl, pH 7.5, 140 mM NaCl)-1% bovine serum
albumin (BSA). Blots were incubated with antibody
30   diluted 1:200 in TBS-1% BSA at room temperature for 1
h. After three washes in TBS containing 0.05% Tween
20, seroreactive proteins were detected with goat
anti-mouse (or rabbit) IgG coupled to alkaline
phosphatase (Kirkegaard & Perry Laboratories, Inc.) at
35   1:5,000 in TBS-1% BSA. Alkaline phosphatase activity
was detected using the Nitro Blue Tetrazolium-5-bromo-

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4-chloro-3-indolylphosphate toluidinium system as
described by the supplier (Promega).

Recombinant DNA techniques.

5   Plasmid DNA was purified as described by
Sambrook et al., *supra*. When required, DNA fragments
were isolated from agarose gels using a Gene Clean kit
(Bio/can Scientific).

          To construct a gene library for *S. uberis*,
10   chromosomal DNA was prepared as previously described
(Caparon and Scott (1987) *Proc. Natl. Acad. Sci. USA*
84:8677-8681) and partially digested with *Sau3AI*.
Fragments of 2,000 to 5,000 bp were recovered
following sucrose density gradient centrifugation
15   (Sambrook et al., *supra*). The ends of these fragments
were partially filled in with dGTP and dATP and
ligated into pTZ18R which was cut with *SalI* and
partially filled in with dTTP and dCTP.
Transformation of *E. coli* DH5α competent cells was
20   carried out as recommended by the supplier (GIBCO BRL,
Gaithersburg, Md). To identify Lf-binding clones,
transformants were replica-plated onto nitrocellulose
discs (Schleicher & Schuell, Keene, NH) and lysed in
chloroform vapor. Nonspecific binding was blocked by
25   incubation with TBS-1% BSA. Membranes were further
incubated with ^{125}I-bLf as described in Example 1.

          Restriction endonucleases, T4 DNA ligase,
DNA polymerase I Klenow fragment and calf intestinal
alkaline phosphatase were utilized according to the
30   manufacturer's directions (Pharmacia Canada Ltd.,
Quebec, Canada).

          DNA sequences were determined by the
dideoxy-chain termination method of Sanger et al.
(1977) *Proc. Natl. Acad. Sci. USA* 74:5463-5467 on
35   double-stranded plasmid templates by using a T7
Sequencing kit (Pharmacia Canada Ltd.).

RNA analyses.

RNA from *E. coli* strains was isolated as described previously (Lloubes et al. (1986) *Nucleic Acid Res.* 14:2621-2636) with an additional RNase-free

5 DNase I digestion.

RNA from *S. uberis* was prepared as follows. The cell pellet from a 10 ml culture ($OD_{600}=0.6$) was resuspended in 250 μl of TE buffer (pH 8.0) containing 500 u of mutanolysin (Sigma) and incubated at 37°C for

10 30 min. Lysis buffer (250 μl) (60 mM Tris-HCl pH 7.4, 200 mM NaCl, 10 mM EDTA, 2% SDS) and 100 μg/ml (final concentration) of proteinase K was added and the incubation continued for 1 h. The sample was extracted once with 65°C phenol (water saturated, pH

15 4.0) and twice with room temperature phenol. RNA was recovered by ethanol precipitation and treated with DNase I (Pharmacia Canada Ltd.).

Northern blot analysis was carried out as described by Sambrook et al., *supra*.

20

Results
Cloning and expression of the *lbp* gene.

A gene library was constructed in pTZ18R with chromosomal DNA from *S. uberis* (su-1). About

25 5000 transformants were initially screened for expression of bLf binding protein (Lbp) by colony blotting with ^{125}I-bLf. One colony with the strongest signal and six with weaker signals were selected and used to make whole cell lysates, which were further

30 tested for their ability to bind ^{125}I-bLF under non-reducing conditions. The clone with the strongest signal, *E. coli* pLBP5, generated three major bands; two of them had molecular weights of 165 and 76 kDa which are quite close in size to those of *S. uberis*.

35 A band slightly larger than 165 kDa was also observed which could be the precursor form with the uncleaved

signal peptide. No corresponding band was found from
the whole cell lysate of *E. coli* pTZ18R, the host
strain control. The recombinant Lbp was also detected
from the periplasm and supernatant of *E. coli* pLBP5,

5   but not from outer or inner membranes, indicating that
the Lbp expressed in *E. coli* DH5α was not membrane-
localized, but instead could be secreted from the
cell.

The presence of free and functionally active

10   recombinant Lbp in the cell lysate and supernatant of
E. coli pLBP5 was also detected by performing
competitive inhibition assays. The *E. coli* pLBP5 cell
lysate and supernatant mixture effectively inhibited
^{125}I-bLf binding to *S. uberis* cells in a dose-dependent

15   manner, while samples from *E. coli* pTZ18R did not
(Figure 11).

To demonstrate whether the protein with a
molecular weight of 165 kDa was a dimer of the 76 kDa
molecule, samples treated with 2-mercaptoethanol or

20   urea were analyzed by Western blotting using rabbit
antiserum against the supernatant of *E. coli* pLBP5.
Mercaptoethanol treatment was shown to have no effect
on the electrophoretic mobility of Lbp from either *S.
uberis* or recombinant *E. coli* while urea-treatment

25   resulted in disappearance of the 165 and 76 kDa bands
and appearance of a new band with an apparent
molecular weight of 105 kDa. This data indicates that
the 165 kDa protein is likely a dimer of the 76 kDa
subunit. The dimer could have been disassociated by

30   urea to monomers which could be further denatured and
unfolded, resulting in the apparent molecular weight
increase from 76 kDa to 105 kDa. A significant
increase in the apparent molecular weight of Tbp2
following urea-treatment has been observed in

35   *Neisseria meningitidis* by other researchers (Vonder
haar et al. (1994) *J. Bacteriol.* <u>176</u>:6207-6213). The

above data also indicates that disulfide bonds are not essential for the formation of the oligomer.

Nucleotide sequence determination and analysis.

5   To determine the nucleotide sequence of the Lbp gene, each of the *HincII*, *HindIII*, *SacI*, *SphI* and *XbaI* fragments of pLBP5 (Figure 1) was individually cloned into the corresponding site of pTZ18R. By using universal and reverse primers, each fragment was

10   sequenced in both orientations. Subsequent primers were synthesized on the basis of sequence information thereby obtained.

Two open reading frames were found from pLBP5 (Figure 1). One was the Lbp-encoding gene *lbp*,

15   whose presence in subclone pLBP5L resulted in a bLf-binding phenotype. The other ORF on the complementary DNA strand was incomplete. A GenBank database search showed that the presumed ORF gene product had significant homology to the VirR and Mry positive

20   regulators in group A streptococci. Thus, this ORF was named *mga'* (Figure 1). The cloning and sequencing of the complete *mga* gene is discussed further below.

The *lbp* sequence contained two potential translation start codons (ATG) at positions 232 and

25   262 of the DNA sequence. Both are associated with putative Shine-Dalgarno sequences (Figures 2A-2C). These start points would give proteins with predicted sizes of 62.857 and 61.454, respectively. The predicted sizes are comparable to 76 KDa molecular

30   weight protein. The reason for the discrepancy between the observed and calculated molecular weights of this protein is not clear. Posttranslational modification, such as lipid modification might have occurred and increased the apparent molecular mass in

35   the SDS-PAGE determination. A similar difference observed in Gram-negative bacteria has been

demonstrated to be caused by protein lipid
modification (Theisen et al. (1992) *Infect. Immun.*
60:826-831; Theisen et al. (1993) *Infect. Immun.*
61:1793-1798).

5   The DNA sequence shows two putative -10 and
-35 promoter regions present at -88 and -102 from the
first ATG. Downstream of *lbp* there is a potential
.rho-independent transcription terminator (Figures 2A-
2C).

10   Analysis of the N-terminus of the predicted
sequence of Lbp showed amino acids characteristic of
signal sequences (Simonen and Palva (1993) *Microbiol.*
Rev. 57:109-137) (Figures 2A-2C). The features of the
sequence are a positively charged N-terminus, rich in
15   K and R residues, followed by a hydrophobic domain
from amino acids 25 to 48 (Figure 3) and the signal
peptidase cleaving site, VKA, at positions 49 to 50,
where cleavage occurs after the A residue. The
presence of this putative signal sequence indicates
20   that the Lbp is exported across the cytoplasmic
membrane of *S. uberis*.

          A search of the GenBank database revealed
that the C-terminus of the Lbp was highly homologous
to the C- terminal ends of streptococcal M proteins,
25   plasminogen binding protein, fibrinogen and IgG-
binding proteins. It shows all the general features
well established for surface proteins of Gram-positive
cocci (Fischetti et al. (1991) Common characteristics
of the surface proteins from Gram-positive cocci, p.
30   290-294. In G. M. Dunny, P. P. Cleary, and L. L. McKay
(ed), *Genetics and molecular biology of streptococci,*
lactococci, and enterococci. American Society for
Microbiology, Washington, D. C.). These features
include a small cluster of four charged amino acids at
35   the C-terminus followed by a hydrophobic domain of 21
amino acids (Figures 2A-2C and Figure 3). Adjoining

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the hydrophobic domain is the consensus membrane
anchor motif LPSTGD. The next region of 50 amino
acids is the cell wall-associated region characterized
by a high proportion of proline and glycine (12%).

5   GenBank database searches also showed that Lbp is
homologous to mammalian myosin heavy chain and kinesin
heavy chain; it has 48% and 46% overall sequence
homology with these two fibrillar proteins,
respectively.

10   The region beyond the membrane anchor motif
and proline/glycine rich region contains three blocks
of amino acids which were found to have internal
homologies (Figures 2A-2C). The A1 block contains 52
amino acids homologous to the A2 block; the B1 block

15   contains 13 amino acids homologous to the B2 block;
and the 59 amino acid C1 block is homologous to the C2
block.

          Analysis of the secondary structure of the
translated protein showed an extensive α-helix region

20   stretching from the proline/glycine-rich region to the
β-sheet and turn region near the cleavage site of the
signal sequence (Figure 3).

Localization of the Lf-binding domain of Lbp.

25   To localize the Lf-binding domains of Lbp,
gene deletions were constructed (Figure 1). 3'-
deletions, pTP31, pTP32, pTP33 and pTP34 were
generated at the restriction enzyme sites HincII,
StuI, XbaI and XmnI of pLBP5L. A 5'-deletion pTP51

30   was constructed by removing the 643 bp HindIII-XbaI
fragment of pLBP5L. All these deletions contained the
lbp promoter. The resulting truncated Lbps were
produced in E. coli and were visualized after SDS-
PAGE, transfer to nitrocellulose, and reaction with

35   specific rabbit antiserum or ^{125}I-bLf. All the samples
were tested under non-reducing conditions in these

experiments. The bLf binding results are summarized in Figure 1. Like full-length Lbp, truncated proteins from *E. coli* pTP31, pTP32 and pTP33 could bind bLf in both monomeric and dimeric forms. Some degradation

5   products from these clones also possessed binding ability. No band was found from *E. coli* pTP34 or pTP51. These data indicate that the primary domain of Lbp involved in the binding of bLf resides in a 22 kDa N-terminal fragment.

10

Cloning and sequencing of the complete *mga* gene.

As described above, the plasmid pLBP5 contained an incomplete gene, *mga'*, in addition to *lbp*. The pLBP5 molecule was inverted to pLBP5i

15   (Figure 4) for convenience of description.

To obtain the complete *mga* gene, a 991 bp *Sty*I fragment from *mga'* was radioactively (^{32}P) labelled as a probe (VP1 in Figure 4) and used to screen an *S. uberis* gene library. A positive *E. coli*

20   DH5α clone was obtained and the restriction enzyme map of the plasmid pMGA14 is shown in Figure 4. To determine the nucleotide sequence of pMGA14, each of the *Bam*HI, *Hinc*II, *Hind*III and *Kpn*I restriction fragments of pMGA14 (Figure 4) was individually cloned

25   into the corresponding site of pTZ18R and sequenced from both orientations using universal and reverse primers. Subsequent primers were synthesized on the basis of sequence information thereby obtained. Plasmid pMGA14 contained the complete ORF of Mga.

30   However, the associated promoter region was not present. Since the 1.5 kb *Sph*I-*Nhe*I fragment of pLBP5i contained the majority of the 5' region of the *mga'* and the complete promoter region, it was inserted into the *Sph*I and *Nhe*I sites of pMGA14 to generate pMGA14F

35   which then contained the complete *mga* gene including the promoter (Figure 4).

The sequence of a 3558 bp DNA fragment of pMGA14F is presented in Figures 5A-5D. Starting at the ATG initiation codon at nucleotides 361-363 and terminating at a TAA codon at nucleotides 1858-1860,

5 the deduced gene product, Mga, is comprised of 499 amino acid residues with a calculated molecular weight of 58,454 Da. The N- terminus of Mga lacks the features of signal peptides as described (Simonen and Palva (1993) *Microbiol. Rev.* 57:109-137), suggesting

10 that it is a cytoplasmic protein. Preceding the start codon of *mga* is a putative ribosome binding site AGGAGA. Sequences resembling the -35 and -10 promoter motifs have also been identified.

A search of the GenBank database revealed

15 that the deduced protein of *mga* has 34% overall sequence identity to the VirR and Mry proteins (Chen et al. (1993) *Mol. Gen. Genet.* 241:685-693); Perez-Casal et al. (1991) *J. Bacteriol.* 173:2617-2624). Studies have shown that these positive regulators of

20 group A streptococcal M proteins contain helix-turn-helix DNA binding domains, and it is believed that via these domains, the regulators may interact directly with specific DNA sequences to influence transcriptional activity (Chen et al., *supra*; Perez-

25 Casal et al., *supra*). Attempts to identify a similar DNA-binding domain with visual inspection and analysis with PCGENE software failed to detect such a motif in Mga of *S. uberis*. However, a region at amino acid residues 106 to 125 (Figures 5A-5D) showed 90%

30 identity to the sequence of the DNA-binding domain of VirR49 (Podbielski et al. (1995) *Infect. Immun.* 63:9-20). Following *mga*, there are four more ORFs with 181, 85, 78 and 128 amino acid residues, respectively (Figures 5A-5D). No significant sequence

35 alignments were found from GenBank.

Northern blot analysis of the *lbp* and *mga* transcripts.

        To analyze the *lbp* and *mga* transcripts, RNA
was prepared from *S. uberis* (su-1), *E. coli*
DH5α(pLBP5), *E. coli* DH5α(pLBP5L), *E. coli*

5   DH5α(pMGA14F) and *E. coli* DH5α(pTZ18R), and used for
two Northern blots. One blot was probed with the 1.5
kb *Hind*III-*Hpa*I internal fragment of *lbp* (LP1 in
Figure 6). A 2.0 kb band was seen in lanes containing
RNA from *S. uberis*, *E. coli* DH5α(pLBP5) and *E. coli*

10   DH5α(pLBP5L), but was absent in the sample which
contained RNA from *E. coli* DH5α(pTZ18R). This data
indicates that only one major transcript was generated
by *lbp* in recombinant *E. coli* as well as in native *S.
uberis*. The second blot was probed with the 1.0 kb

15   *Nco*I-*Nhe*I internal fragment of *mga* (VP2 in Fig. 4). A
1.8 kb band was seen in samples which contained RNA
from *E. coli* DH5α(pLBP5) and *E. coli* DH5α(pMGA14F),
respectively. No band was found from *S. uberis* and *E.
coli* DH5α(pTZ18R) RNA samples. The *mga* and *mga'* genes

20   were transcribed in recombinant *E. coli*, and the stop
codon of the vector must have been utilized during the
transcription of *mga'* gene. However, it is unclear
whether the absence of a visible hybridization band
from *S. uberis* was due to the low quantity of the gene

25   transcript or the inactivity of the *mga* gene.

Southern blot analysis of the *lbp* and *mga* distribution
in *S. uberis* strains.

        A collection of *S. uberis* including five

30   ATCC strains and 42 field isolates (Table 2) was used
in Southern hybridization experiments. Chromosomal
DNA was prepared and digested with the restriction
endonuclease *Hind*III and separated on agarose gels.
To analyze the *lbp* gene distribution, a 1.5 kb

35   *Hind*III-*Hpa*I fragment which contained the most DNA
sequence from the region encoding Lbp (LP1 in Figure

6) was used as a probe. This probe hybridized with DNA
from 42/47 strains (Table 2). This result meant
either that *lbp* shared a region of homology with most
of the *S. uberis* strains or that among these strains,

5   there were different regions of the *lbp* gene
homologous to the *lbp* probe. To locate the homologous
region(s) more specifically, this *lbp* probe was
subdivided into smaller segments (Figure 6). Two
probes, the 643 bp *Hind*III-*Xba*I fragment (LP2) and the

10   437 bp *Xba*I-*Stu*I fragment (LP3) covering the N-
terminal and central portion of the coding region in
the *lbp* gene, respectively, hybridized only with su-1,
the strain from which the *lbp* gene was originally
cloned, whereas the 409 bp *Stu*I-*Hap*I fragment from the

15   C-terminal region (LP4) hybridized with all the
strains that were hybridized by probe LP1 (Table 2).
It appears to be clear that the region of the *lbp* gene
encompassing the coding sequences for part of the C
repeat, the proposed wall attachment region and the

20   membrane anchor, is conserved among *S. uberis* strains,
while the region encoding the N-terminal portion
varies greatly. The diversity in sizes of chromosomal
restriction fragments from the different strains that
hybridized with the *lbp* probes indicates some degree

25   of restriction site heterogeneity (a different
location for *Hind*III site) among these strains.

30

35

Table 2. *S. uberis* strains and DNA hybridization with LP
and VP probes

Strain	Source[a]	Probe				
		LP1	LP2	LP3	LP4	VP3
su-1	ATCC9927	3.40[b]	3.40	3.40	3.40	0.87
su-2	ATCC13386	-[c]	-	-	-	-
su-3	ATCC13387	3.60	-	-	3.60	3.60
su-4	ATCC19436	2.70	-	-	2.70	1.70
su-5	Chirino 93-1869	-	-	-	-	-
su-6	Chirino 93-2017	-	-	-	-	-
su-7	Chirino 93-8678-2	4.40	-	-	4.40	4.40
su-8	Chirino-*S. uberis*	5.00	-	-	5.00	0.91
su-9	ATCC27958	3.80	-	-	3.80	0.87
su-10	Greenfield 93-4997	3.20	-	-	3.20	1.50
su-11	Greenfield 93-4997	3.20	-	-	3.20	1.50

Table 2. (continued)

Strain	Source[a]	Probe				
		LP1	LP2	LP3	LP4	VP3
su-12	Leduc	3.80	-	-	3.80	0.87
su-13	Leduc	3.80	-	-	3.80	0.87
su-14	Alix	5.00	-	-	5.00	5.00
su-15	Magrath	4.10	-	-	4.10	0.90
su-16	Ohaton	5.70	-	-	5.70	0.90
su-17	Winfield	2.90	-	-	2.90	1.40
su-18	Not known	4.10	-	-	4.10	1.00
su-19	Gibbons	3.60	-	-	3.60	1.20
su-20	Ardrossan	4.40	-	-	4.40	4.40
su-21	Not known	3.40	-	-	3.40	1.00
su-22	Dapp	2.90	-	-	2.90	1.42
su-23	Thorsby	3.20	-	-	3.20	1.40
su-24	Ponoka	3.80	-	-	3.80	0.87
su-26	Warburg	4.40	-	-	4.40	0.90
su-27	Leduc	-	-	-	-	-
su-28	Fort MacLeod	3.00	-	-	3.00	1.80
su-29	Sherwood Park	5.00	-	-	5.00	5.00
su-30	Barrhead	5.00	-	-	5.00	5.00
su-31	Wainwright	5.00	-	-	5.00	5.00
su-32	Tofield	3.60	-	-	3.60	1.03
su-33	Barrhead	5.00	-	-	5.00	5.00
su-34	Barrhead	5.00	-	-	5.00	5.00
su-35	Medicine Hat	5.00	-	-	5.00	5.00
su-36	Ponoka	5.00	-	-	5.00	5.00
su-37	Ponoka	5.00	-	-	5.00	5.00
su-38	Ponoka	5.00	-	-	5.00	5.00
su-39	Ponoka	5.00	-	-	5.00	5.00
su-40	Millet	3.60	-	-	3.60	1.03
su-41	Lacombe	3.00	-	-	3.00	1.40
su-42	Daysland	5.00	-	-	5.00	5.00
su-43	Ohaton	5.30	-	-	5.30	0.97
su-44	Ohaton	5.00	-	-	5.00	5.00
su-45	Didsbury	-	-	-	-	-
su-46	Didsbury	4.70	-	-	4.70	4.70
su-47	Didsbury	5.00	-	-	5.00	5.00
su-48	Didsbury	4.70	-	-	4.70	4.70

[a] All strains are field isolates except five ATCC
(American Type Culture Collection) strains.
[b] Size of hybridizing fragment (in kilobase).
[c] Lack of hybridization.

In order to study whether there was an *mga*-
related gene in other *S. uberis* strains, the previous
blot was stripped and reprobed with a 572 bp *Hind*III

fragment that encompassed the 5'-region of the *mga*
gene (VP3 in Figure 4). Hybridization was detectable
in all *S. uberis* strains that showed homology with the
lbp gene (Table 2). The specific band which reacted
5   with the *mga* probe in each strain is listed in Table
2.

<div align="center">Discussion</div>

          The Lbp gene of *S. uberis* was cloned in *E.*
10   *coli* after screening a gene library by colony blotting
with ^{125}I-bLf. *E. coli* transformants produced
functionally active bLf-binding proteins with
molecular weights of 76 kDa and 165 kDa, similar to
the native proteins produced by *S. uberis*.
15   Considering the one-affinity binding phenomenon of bLf
with *S. uberis* (Example 1), we wondered whether the
165 kDa protein was a dimer of the 76 kDa molecule.
Treatment with the reducing reagent β-mercaptoethanol
did not have any effect on the mobilities of these
20   proteins, indicating the absence of disulfide bridges
in the protein structure. This was confirmed after
the sequencing data was available; there are no
appropriate cysteine residues within this protein.
After boiling for 30 min in the presence of 3 M urea,
25   proteins were completely denatured. This treatment
resulted in a significant molecular weight decrease of
the 165 kDa protein, probably resulting from the
dissociation of the dimer, and an increase in
molecular weight of the 76 kDa resulting from protein
30   unfolding. The dimer structure seems to be very
stable, since treatment with lower concentration of
urea or shorter boiling time could not alter the
mobility of the 165 kDa band. The presence of both
monomeric and dimeric Lbp in *S. uberis* cell wall
35   preparations may imply the existence of both forms on
the bacterial surface. However, it is possible that

only the dimeric form exists on the bacterial cell;
the monomer might come from the cytoplasm due to
partial cell lysis caused by glass bead treatment.
The dissociation of the dimer to a monomer during
5   sample preparation seems unlikely because of the
stability of the dimer.

The complex nature of the *S. uberis* Lbp was
further confirmed from the nucleotide sequence
analysis. As expected, the two Lbps were encoded by a
10   single ORF of 1,683 bp. It is quite possible that two
copies of the translated product interact with each
other to form a homodimer. Interactions between
subunits could be extensive and extend throughout the
length of the molecule due to the existence of the
15   high α-helical content. Like M proteins (Fischetti,
V.A. (1989) *Clin. Microbiol. Rev.* 2:285-314), Lbp
shares significant sequence homology with a number of
α-helical coiled structure-containing mammalian
fibrillar proteins such as human myosin heavy chain
20   and kinesin heavy chain.

The deduced amino acid sequence of Lbp
indicated the existence of a signal peptide of 50
amino acids (if translation started at the first ATG
start codon) at the N-terminus, as expected for a
25   protein that appears on the outside of a bacterial
cell. The structure of this signal peptide is
comparable to the consensus structures for signal
peptides in proteins from Gram positive bacteria
described previously (Simonen and Palva (1993)
30   *Microbiol. Rev.* 57:109-137; Goward et al. (1993)
Trends Biochem. Sci. 18:136-140; Perlman and Halvorson
(1983) *J. Mol. Biol.* 167:391-409; von-Heijne, G.
(1983) *Eur. J. Biochem.* 133:17-21; von-Heijne, G.
(1986) *Nucleic Acids Res.* 14:4683-4690).

35   Analysis of the C-terminus of Lbp revealed
the presence of an amino acid sequence typical of

membrane anchor motifs found in many other proteins
from Gram- positive bacteria (Fischetti et al. (1991)
Common characteristics of the surface proteins from
Gram-positive cocci, p. 290-294. In G. M. Dunny, P. P.

5   Cleary, and L. L. McKay (ed), *Genetics and
molecular biology of streptococci, lactococci, and
enterococci*. American Society for Microbiology,
Washington, D. C.). Such regions have been shown to
play a role in the anchoring of proteins in the cell

10   wall (Pancholi and Fischetti (1988) *J. Bacteriol.*
170:2618-2624; Schneewind et al. (1992) *Cell* 70:267-
281; Schneewind et al. (1993) *Embo. J.* 12:4803-4811).
It is probably via the C-terminal amino acids that Lbp
becomes anchored on the cell surface. The membrane

15   anchor motifs described here appear to have no
function in *E. coli* since the Lbp expressed in *E. coli*
was mainly secreted, similar to the streptococcal M6.1
protein which was found predominantly in the periplasm
(Fischetti et al., 1984).

20   5'- and progressive 3'-deletions to the *lbp*
gene allowed the definition of a large domain (about
200 codons) at the N-terminus which could bind bLf. A
comparison of this region with the transferrin binding
domain of Tbp2 from *Neisseria* (Vonder Haar et al.

25   (1994) *J. Bacteriol.* 176:6207-6213) showed no
significant homology. Also, the bLf binding region
reported here did not contain any of the domains with
transferrin binding activity found in the transferrin-
binding protein (TfbA) of *Actinobacillus*

30   *pleuropneumoniae* (Strutzberg et al. (1995) *Infect.
Immun.* 63:3846-3850). This is not surprising since,
as shown in Example 1, bovine or human transferrin
could not block the binding of bovine lactoferrin to
S. uberis. This indicates a difference between the

35   bLf-binding domain and the Tf-binding domain.
A second ORF, *mga*, was found adjacent to the *lbp* gene

region. The putative product of this gene (containing
499 amino acid residues) has comparable molecular size
and sequence to VirR12 (499 residues) and Mry (530
residues), the positive regulators of the M protein

5   genes of group A streptococci (Chen et al. (1993) *Mol.
Gen. Genet. 241:685-693); Perez-Casal et al. (1991) *J.
Bacteriol.* 173:2617-2624). However, Mry and VirR12
had 98% homology (Chen et al., *supra*), whereas Mga
showed only 34% homology with them. This may be

10 attributed to the difference of species. Similarly,
VirR49 of an OF⁺ GAS showed less homology (76%) to Mry
or VirR of OF⁻ GAS (Podbielski et al. (1995) *Infect.
Immun.* 63:9-20).

        The cytoplasmic location of Mga was

15 suggested by the absence of a signal peptide at the N-
terminus of the deduced protein. A potential -10 and
-35 promoter was found.

        Studies have shown that *mry* is autoregulated
and environmentally regulated in response to the level

20 of CO_2 (Okada et al. (1993) *Mol. Microbiol.* 7:893-903).
Expression of *mry* was stimulated by increased
concentrations of CO_2. In our experiments, *S. uberis*
cells were cultured under conditions without an
additional supplementation of CO_2. The absence of a

25 stimulating environment could have resulted in a very
low level of *mga* expression. This could be the reason
that Northern blot analysis did not detect any *mga*
transcript from *S. uberis*. It would be expected that
in recombinant *E. coli*, *mga* would not likely be

30 regulated by environmental signals due to the absence
of other regulatory components such as the sensing
protein.

        It would be interesting to know whether *mga*
regulates the expression of *lbp*. No potential VirR-

35 binding boxes that are present in the promoters of M
and M-like protein genes (Podbielski et al. (1992)

Mol. Microbiol. <u>6</u>:2253-2265; Podbielski et al. (1995)
Infect. Immun. <u>63</u>:9-20) were found in the immediate
upstream portion of the -35 region of *lbp* by homology
comparisons. 47 *S. uberis* strains were blotted with

5   *mga* and *lbp* specific probes in order to determine the
distribution of *mga* and its relation to *lbp* in this
streptococcal group. Results showed that all strains
that were *lbp* positive contained *mga*.

        Southern blot analysis of *lbp* in *S. uberis*

10   strains using subgenomic probes revealed that the C-
terminal sequence is conserved among strains, whereas
the N-terminal region shows greater variation. This
phenomenon resembles that described for M protein
genes in group A streptococci. By using DNA

15   hybridization with probes from the structural gene for
the M6 protein, Scott et al. (1986) *Infect. Immun.*
<u>52</u>:609-612 showed that the C-terminal region is highly
conserved among strains of different M serotypes and
the N-terminal region is highly variable. Consistent

20   with this, detailed amino acid sequence comparisons
and antibody reactivities revealed limited differences
within the conserved C-terminal regions and much more
extensive variability at the N- termini (Bessen and
Fischetti (1990) *J. Exp. Med.* <u>172</u>:1757-1764; Bessen et

25   al. (1989) *J. Exp. Med.* <u>169</u>:269-283; Kehoe, (1991)
Vaccine <u>9</u>:797-806). In the case of Lf binding
proteins of *S. uberis*, however, limited sequence and
serological data are available for comparisons. In M
proteins, the N-terminal region is distal to the

30   streptococcal cell surface, and thus would be expected
to be the region of the molecule most exposed to
immunological selective pressure. Therefore it is not
surprising that the N-terminal region varies in
sequence among different serological types of M

35   protein. In contrast, the sequence of the C-terminal
region of the molecule should be evolutionarily

conserved to assure attachment to the streptococcal
surface. Since Lbp has a structure similar to that of
M protein, it is not surprising to find that its C-
terminal sequence is conserved among strains, whereas

5   the N-terminal region shows variation.

        Since the N-terminal part of the Lbp is
variable among *S. uberis* strains, our finding that
this part of the molecule is responsible for Lf
binding seems surprising. Similar observations have

10   been reported in *Actinobacillus pleuropneumoniae*.
Three isoforms of transferrin binding proteins (Tbp2)
from different *A. pleuropneumoniae* serotypes contain a
variable N-terminal half and a conserved C-terminal
half (Bunka et al. (1995) Cloning and sequencing of

15   the transferrin-binding protein genes of
Actinobacillus pleuropneumoniae, biotype 1-serotype 5
and biotype 2-serotype 2. Unpublished; GenBank
accession no. Z46774; Gerlach et al. (1992) *Infect.
Immun.* 60:3253-3261; Gerlach et al. (1992) *Infect.*

20   *Immun.* 60:892-898); the N-terminal half of the
molecule is responsible for transferrin binding
(Strutzberg et al. (1995) *Infect. Immun.* 63:3846-
3850).

25   Example 3
        Evaluation of the Protective Capacity of
       Recombinant Lactoferrin-Binding Protein against
                  Challenge by *S. UBERIS*

30   Materials and Methods
        Bacterial strains, vectors and media.
        S. uberis strain su-1 (ATCC 9927) was
obtained from a clinical case of bovine mastitis.
Bacterial cells were grown in tryptic soy broth,

35   aliquoted and stored at -70°C on blood beads until
needed. *E. coli* strain DH5α was used in all

transformation experiments. *E. coli* cells were
cultured in Luria medium, and the medium for the
growth of transformants was supplemented with 50 µg/ml
of ampicillin. The plasmid pGH433 (Anderson et al.
5   (1991) *Infect. Immun.* <u>59</u>:4110-4116) was used to
express the recombinant proteins under the control of
an IPTG (isopropyl β-D-thiogalacto-pyranoside)-
inducible promoter.

10 <u>PAGE and Western blotting.</u>
          Lbp inclusion bodies were dissolved in
sample buffer in the presence of 4 M urea and run on
an SDS-PAGE gel containing 4 M urea. Western blotting
was done using convalescent serum from an su-1-
15 infected cow using a 1:75 dilution of cow convalescent
serum and a 1:5000 dilution of goat anti-bovine IgG
coupled to alkaline phosphatase.
<u>Protein purification.</u>
          A culture of *E. coli* transformants (1 L) was
20 grown to an absorbance at 660 nm of 0.5 and induced
with 2 mM IPTG. After 2 h of continuous, vigorous
shaking at 37°C, the cells were harvested and the
protein inclusion bodies were prepared as described by
Gerlach et al. (1992) *Infect. Immun.* <u>60</u>:892-898. A
25 mid-log phase broth culture (1 L) grown at 30°C was
cultured at 42°C for 2 h with vigorous shaking for
protein induction. Cells were harvested by
centrifugation, resuspended in 5 ml of 25% sucrose-50
mM Tris-HCl buffer (pH 8.0), and frozen at -70°C.
30 Lysis was achieved by the addition of 1 mg of lysozyme
in 250 mM Tris-HCl buffer (pH 8.0), 10 min of
incubation on ice, addition of 25 ml of a detergent
mix (5 parts of 20 mM Tris-HCl buffer, pH 7.4, 300 mM
NaCl, 2% deoxycholic acid, 2% Nonidet P-40 and 4 parts
35 of 100 mM Tris-HCl buffer, pH 8.0, 50 mM EDTA, 2%
Triton X-100), and sonication. Inclusion bodies were

harvested by centrifugation for 30 min at 15,000 x g
and resuspended in H_2O to a concentration of 5 to 10
mg/ml. Antigens for ELISA were purified from SDS-PAGE
gels or 4 M urea SDS-PAGE gels by elution.

5

Determination of protein purity and concentration.

Protein purity was estimated by SDS-PAGE and
subsequent Coomassie blue staining. The protein
concentration was determined using Bio-Rad DC protein
10   assay as described by the supplier. Bovine serum
albumin (Pierce Chemical Co., Rockford, IL) was used
as a standard. The amount of target protein vs. total
protein was determined after scanning the Coomassie
blue stained
15   SDS-PAGE with a Bio-Rad 620 Densitometer.

Vaccine preparation and vaccination.

The vaccines consisted of proteins
emulsified in the adjuvant VSA3 which had been diluted
20   with 0.1 M PBS, pH 7.2. Each 2 ml dose of vaccine
contained VSA (0.67 ml), PBS (1.33 ml), and 50 μg of
protein antigen dissolved in 5-10 μl of 4 M guanidine
hydrochloride. Fifteen healthy lactating dairy cows
-from the Pennsylvania State University Mastitis
25   Research Herd were vaccinated intramuscularly at
drying off and again 28 days later. Groups of five
cows were given Lbp or adjuvant only.

Challenge.

30   The bacterial challenge culture was prepared
by rolling the stock bead cultures onto esculin blood
agar plates containing 5% whole blood. After 24 hours
incubation at 37°C, a single colony was used to
inoculate 100 ml of Ultra High Temperature pasteurized
35   (UHT) milk and incubated for 12 hours at 37°C. The 24
hour culture was mixed well and a 100 μl aliquot was

removed to inoculate a second 100 ml of UHT milk.
After a second 9 hour incubation at 37°C, the culture
was serially diluted in 10-fold increments using
sterile saline. The colony forming units (CFU) per ml

5   of each dilution was determined by absorbance on a
spectrophotometer and confirmed by plating onto blood
agar. Animals were challenged by intramammary
infusion of 200 CFU of *S. uberis* in 1 ml of saline in
the teat canal of one quarter on day four of

10   lactation.

Sampling.

        Milk and blood samples were obtained at the
times outlined in Table 3.

15

Table 3. Immunization, challenge and Sampling
schedule.

TIME	SAMPLE
dry off, D-0	serum, milk, immunization
14 days dry, D+14	serum
28 days dry, D+28	serum, immunization
52 days dry, D+52	serum
calving, C-0	serum, milk
4 days lactation, CH-0	serum, milk, challenge
7 days lactation, CH+3	serum, milk
14 days lactation, CH+10	serum, milk
21 days lactation, CH+17	serum, milk

30   Antibody Titers.

        Total Ig titers for the Lbp antigen were
determined by an indirect ELISA. Nunc Immunlon-2
plates were coated with antigen in carbonate buffer.
Prior to use, the plates were blocked with TBST (100

35   mM Tris-Cl, pH 8.0; 150 mM NaCl; 0.05% Tween-20) and
3% BSA for 1 hour. After blocking, the plates were

washed with distilled water. Serum and milk samples
were serially diluted in 3-fold increments using TBST
containing 1% BSA. Rabbit antisera for the Lbp
antigen was also diluted and served as a positive

5   control. Negative control samples contained TBST with
1% BSA. The diluted samples and controls were
transferred to the coated plates and were incubated
for 1 hour at room temperature. The plates were washed
thoroughly with distilled water and all wells were

10   incubated with a horse radish peroxidase conjugate of
goat anti-IgG diluted 1:2000 in TBST containing 1%
BSA. Following a 1 hour incubation at room
temperature, the plates were washed with distilled
water. The amount of antibody present in samples was

15   visualized using ABTS substrate. The titers of each
sample were based on the absorbance reading at 405 nm
with a reference wavelength of 495 nm. A positive
reading for samples was one in which the absorbance
was two times the absorbance of the blank (negative

20   control). Titers were determined by taking the
reciprocal of the last dilution giving a positive
reading. Consistency among assay plates was monitored
by the absorbance reading of positive controls.

25   Milk compositional analysis.
     Milk pH values, total somatic cell counts,
fat, protein, and lactose were determined using a
Fossomatic Cell and Milk Analyzer (A/S Foss Electric,
Hillerød, Denmark).

30

DNA manipulations.
     All molecular techniques were as recommend
by the supplier (Pharmacia Canada Ltd.) or Sambrook et
al. *Molecular Cloning: A Laboratory Manual*, Second

35   Edition (1989). When required, plasmid DNA fragments

were isolated from agarose gels using a Gene Clean kit
(Bio/can Scientific).

Results

5   Expression of Lbp under the control of the tac
     promoter.

          Plasmid pGH-LBP was constructed by inserting
     the 1.8 kb SphI-RsaI fragment from pLBP5 into the
     BamHI site of pGH433 (Fig. 10) which provides a 12
10   amino acid leader peptide and an IPTG-inducible tac
     promoter. Before ligation, the insert was treated
     with mung bean nuclease to remove the 3' overhang from
     the end generated by SphI, and the BamHI-cut vector
     was filled in by the Klenow fragment to produce blunt
15   ends. The pGH-LBP contained the carboxy-terminal 96%
     of the Lbp gene (lbp), which was preceded by a 12
     amino acid leader peptide and the tac promoter
     provided by vector pGH433. Analysis of the nucleotide
     sequence at the fusion site revealed identity to the
20   sequence of the lbp presented in Figure 1.

     Purification of recombinant proteins.

          Lbp of S. uberis was expressed in E. coli DH5α
     using pGH-LBP. In this system, expression of the
25   recombinant protein was repressed under normal growth
     conditions. Upon IPTG-induction, the recombinant
     protein was produced in an aggregated form. The 82
     and 90 kD Lbp made up 36% of the total protein.
     Isolated protein aggregates were dissolved in 4 M
30   guanidine hydrochloride and used for vaccine
     formulation. Lbp was purified from a 4 M urea SDS-
     PAGE gel and used as the antigen for ELISA. Both
     aggregated and purified Lbp were demonstrated to be
     antigenically active by Western blotting using
35   convalescent serum from S. uberis infected cow.

Somatic cell counts in milk following experimental
bacterial challenge.

The mean somatic cell count in milk from
challenged quarters of nonvaccinated control animals
increased up to 3,000,000 cell/ml 3 days following
challenge. The challenged quarters of animals
vaccinated with Lbp showed a rapid increase in the
mean SCC which was close to that in the control group.

Effect of vaccination of Lbp-specific antibody titers.

The mean levels of antibodies specific to
Lbp in the serum and milk of the control animals were
low prior to experimental challenge. In one trial,
Lbp-specific antibody levels in the serum and milk of
animals vaccinated with Lbp were not significantly
increased following vaccination when compared with
prevaccination levels or levels in the control
animals. In a subsequent trial, an antibody response
was seen.

Changes of pH value and main constituent of milk after
challenge.

The mean pH values of milk from animals
vaccinated with placebo and Lbp changed in a similar
pattern after challenge. The percentage of fat,
lactose and protein in milk from immunized and
unimmunized animals also changed in a similar pattern
after challenge. No obvious decrease of these values
occurred.

Discussion

The surface exposure of *S. uberis* Lbp makes
it a potential vaccine candidate by increasing the
levels of opsonic antibody in milk and potentiating
the speed of PMN recruitment into the mammary gland.
Furthermore, the above example shows that Lbp is

recognized by bovine serum from an animal which
recovered from an *S. uberis* infection, indicating that
the protein is expressed *in vivo* and cows respond to
it immunologically. Based on these considerations,

5   the protective capacity of recombinant Lbp was tested
in lactating dairy cows against challenge by *S.
uberis*.

Lbp was produced as inclusion bodies in the
expression system used, facilitating the purification

10   of large quantities of pure recombinant proteins. In
one study, Lbp did not induce high levels of specific
antibodies in the immunized cows, likely due to the
formulation parameters. In these animals, bacterial
challenge resulted in high somatic cell counts,

15   indicative of mastitis. Therefore, vaccination with
Lbp did not prevent the occurrence of mastitis.
However, the serological response to vaccination was
very poor and therefore it is not possible to draw any
conclusions regarding the protective capacity of Lbp

20   from this study.

In a subsequent study, Lbp elicited an
antibody response in immunized cows. Protection
studies are on-going and preliminary results indicate
that Lbp is effective in protecting subjects from

25   mastitis.

The potential involvement of mammary gland
lymphocytes in the protection against *S. uberis*
·mastitis following vaccination with killed *S. uberis*
via the intramammary or subcutaneous route has been

30   suggested elsewhere (Finch et al. (1994) *Infect.
Immun.* 62:3599-3603). Recent observations have
indicated that protection against mastitis caused by
S. uberis does not appear to be related to levels of
specific antibody. Intramammary or subcutaneous

35   administration of *S. uberis* at drying off has been
shown to dramatically reduce both the incidence of

clinical mastitis and numbers of bacteria recovered
from the milk following challenge with the same strain
during the next lactation (Finch et al., *supra*; 1994;
Hill et al. (1994) *FEMS Immunol. Med. Microbiol.*

5   *8*:109-118). Although there was a significant increase
in the levels of *S. uberis*-specific immunoglobulin
following vaccination, there was no increase in the
opsonic activity of serum and milk. Furthermore, it
has been demonstrated that *S. uberis* can resist the

10   bactericidal activity of neutrophils despite the
presence of Ig bound to the surface of the bacteria
(Leigh and Field (1994) *Infect. Immun.* *62*:1854-1859).
Therefore, assessment of cellular immune response
should be of equal importance to that of antibody

15   response when evaluating a vaccination regime against
S. uberis mastitis.

During our vaccine trial, no obvious
clinical signs of mastitis were observed after
challenge in immunized or unimmunized animals.

20   Therefore, it is not surprising that the pH value and
main constituent of milk were not influenced.

_____

25   Thus, *S. uberis* lactoferrin-binding proteins
are disclosed, as are methods of making and using the
same. Although preferred embodiments of the subject
invention have been described in some detail, it is
understood that obvious variations can be made without

30   departing from the spirit and the scope of the
invention as defined by the appended claims.

35

<u>CLAIMS</u>

1. An isolated immunogenic *Streptococcus uberis* bovine lactoferrin-binding protein.

5

2. The lactoferrin-binding protein of claim 1 comprising an amino acid sequence having at least about 80% identity to the amino acid sequence shown at amino acid positions 1 through 561, inclusive, of

10 Figures 2A-2C (SEQ ID NO:1-2), or an immunogenic fragment thereof comprising at least about 10 amino acids.

3. The lactoferrin-binding protein of claim

15 1 comprising an amino acid sequence having at least about 80% identity to the amino acid sequence shown at amino acid positions 52 through 561, inclusive, of Figures 2A-2C (SEQ ID NO:1-2), or an immunogenic fragment thereof comprising at least about 10 amino

20 acids.

4. An isolated nucleic acid molecule comprising a coding sequence for an immunogenic *Streptococcus uberis* bovine lactoferrin-binding

25 protein.

5. The nucleic acid molecule of claim 4 wherein said molecule comprises a nucleotide sequence having at least about 80% identity to the nucleotide

30 sequence shown at nucleotide positions 232-1914, inclusive, of Figures 2A-2C (SEQ ID NO:1-2), or a fragment thereof comprising at least about 15 nucleotides.

35 6. The nucleic acid molecule of claim 4 wherein said molecule comprises a nucleotide sequence

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having at least about 80% identity to the nucleotide
sequence shown at nucleotide positions 445-1914,
inclusive, of Figures 2A-2C (SEQ ID NO:1-2), or a
fragment thereof comprising at least about 15
5   nucleotides.

7.   A recombinant vector comprising:
(a) a nucleic acid molecule according to any
of claims 4 to 6; and
10   (b) control elements that are operably
linked to said nucleic acid molecule whereby said
coding sequence can be transcribed and translated in a
host cell, and at least one of said control elements
is heterologous to said coding sequence.
15

8.   A host cell transformed with the
recombinant vector of claim 7.

9.   A method of producing a recombinant
20   bovine lactoferrin-binding protein comprising:
(a) providing a population of host cells
according to claim 8; and
(b) culturing said population of cells under
conditions whereby the bovine lactoferrin-binding
25   protein encoded by the coding sequence present in said
recombinant vector is expressed.

10.   A vaccine composition comprising a
pharmaceutically acceptable vehicle and an immunogenic
30   bovine lactoferrin-binding protein according to any of
claims 1 to 3.

11.   The vaccine composition of claim 10,
further comprising an adjuvant.
35

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12.   A method of producing a vaccine
composition comprising:
        (a) providing an immunogenic *S. uberis*
bovine lactoferrin-binding protein according to any of
5   claims 1 to 3; and
        (b) combining said lactoferrin-binding
protein with a pharmaceutically acceptable vehicle.

13.   Use of a bovine lactoferrin-binding
10   protein according to any of claims 1 to 3 for the
manufacture of a composition useful for treating or
preventing mastitis in a mammalian subject.

14.   Antibodies directed against a
15   *Streptococcus uberis* bovine lactoferrin-binding
protein according to any of claims 1 to 3.

15.   The antibodies of claim 14, wherein the
antibodies are polyclonal.
20

16.   The antibodies of claim 14, wherein the
antibodies are monoclonal.

17.   A method of detecting *Streptococcus*
25   *uberis* antibodies in a biological sample comprising:
        (a) providing a biological sample;
        (b) reacting said biological sample with a
S. uberis bovine lactoferrin-binding protein according
to any of claims 1 to 3, under conditions which allow
30   *S. uberis* antibodies, when present in the biological
sample, to bind to said *S. uberis* bovine lactoferrin-
binding protein to form an antibody/antigen complex;
and
        (c) detecting the presence or absence of
35   said complex,

thereby detecting the presence or absence of
S. *uberis* antibodies in said sample.

18. An immunodiagnostic test kit for
detecting *Streptococcus uberis* infection, said test
kit comprising a S. *uberis* bovine lactoferrin-binding
protein according to any of claims 1 to 3, and
instructions for conducting the immunodiagnostic test.

19. An isolated *Streptococcus uberis* bovine
Mga protein.

20. An isolated nucleic acid molecule
comprising a coding sequence for a *Streptococcus
uberis* bovine Mga protein.